Regulation of cell behaviour and identity in a branching epithelium

Derina E Sweeney

Doctor of Philosophy,
University of Edinburgh, 2005

Table of contents

Declaration

I declare that:

(a) this thesis was composed by me;

(b) the work presented here is my own, except where stated; and

(c) the work has not been submitted for any other degree or professional qualification

Derina E Sweeney

List of figures

List of Tables

List of Abbreviations

2D	two dimensional
3D	three dimensional
APS	adenosine 5'-phosphosulfate
ATP	adenosine 5'-triphosphate
Bmp	bone morphogenetic protein
BrdU	bromodeoxyuridine
cDNA	complementary deoxyribonucleic Acid
CHAPS	3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate
DBA	*Dolichos biflorus* agglutinin
DEPC	diethyl pyrocarbonate
dH_20	DEPC treated H_2O
DIG	digoxigenin
DNA	deoxyribonucleic Acid
dPBS	DEPC treated PBS
dPBT	DEPC treated PBT
E	embryonic day
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
Erk	extracellular signal-regulated protein kinase
FGF	fibroblastic growth factor
FITC	fluorescein Isothiocyanate
GAG	glycosaminoglycan
Gdnf	glial derived neurotrophic factor
GFP	green fluorescent protein
Gfrα	glial derived neurotrophic factor receptor alpha
GPI	glycophosphatidyl inositol group
Hgf	hepatocyte growth factor
Ig	immunoglobulin
LB	Laria-Bertani
MAPK	mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney cells
MEM	Eagle's minimum essential medium

mIMCD	mouse inner medullary collecting duct cells
mRNA	messenger ribonucleic acid
NBT	nitro-blue tetrazolium chloride
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-yoluidine salt)
NTP	nucleotide triphosphate
OD	optical density
OPT	optical projection tomography
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PBS	phosphate buffered saline
PFA	paraformaldehyde
Rar	retinoic acid receptor
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
Shh	sonic hedgehog
siRNA	small interfering ribonucleic acid
SSC	saline sodium citrate
TBE	tris-borate-EDTA
TBT	tris-borate-EDTA with 0.1% tween20
Tgf	transforming growth factor
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
TRITC	tetramethyl rhodamine iso-thiocyanate
tRNA	transfer ribonucleic acid

Gene symbol nomenclature:

Human genes are stated by using all uppercase letters and are italicised. Mouse gene and protein symbols are written according to the guidelines of the Mouse Genome Informatics Web Site, outlined at http://www.informatics.jax.org/mgihome/nomen/short_gene.shtml.
Mouse gene symbols begin with an uppercase letter followed by all lowercase letters and are italicised. Protein symbols use all uppercase letters and are not italicised.

Abstract

Branching morphogenesis is critical for the development of many organs including the lung, pancreas, kidney, breast and prostate. In the developing kidney, the branching epithelium is called the ureteric bud; it is divided into tip regions, at its ends, and the stalk regions everywhere else. The tip regions are capable of inducing nephron formation, unlike the stalk regions. Tip regions are also the regions where most branching occurs. The cells of the tip regions of the ureteric bud have the ability to proliferate and differentiate into cells of the stalk region. Although differentiation and morphogenesis of the ureteric bud have been studied for many years, the mechanisms that control their overall pattern remain unknown. In this thesis, I have tested a specific set of hypotheses in which both differentiation and morphogenesis are controlled by a self-organization based on inhibitory interactions between tip and stalk cells.

Using micro dissection and organ culture I show that;

- The ureteric bud is composed of at least two distinct populations of cells, those that bind Dolichos biflorus agglutinin (DBA) and those that do not. These correspond to the stalk and tip regions respectively. DBA is a marker of regions of the ureteric bud in which branching morphogenesis is inactivated. When branching morphogenesis is inactivated a change in cell behaviour of the tip cells of the ureteric bud takes place. Tip regions change to a stalk-like behaviour as they loose the expression of tip-specific markers with a parallel increase in stalk markers.
- Stalk cells are capable of giving rise to tip cells. Using DBA as a marker of stalk cells, I investigate the mechanisms controlling branching of the ureteric bud. Firstly, I tested the hypothesis that branches rarely arise from the stalks of the ureteric bud because they have lost the ability to branch; it seems that the stalk cells retain their ability to become tips when provided with an appropriate environment. Differentiation of ureteric bud cells is therefore surprisingly plastic.
- I also tested the hypothesis that tips of the ureteric bud space out by sensing and responding to other tips in the vicinity. There are two components to this hypothesis;

(i) that tips are separated within the epithelium by a lateral inhibition mechanism that prevents new tips forming close to existing ones, and

(ii) that tips of extending epithelia are repelled by the presence of nearby tips, so that they spread out to fill space optimally.

I have gained evidence against the first hypothesis and show that tips can form from stalk regions. I have investigated the second hypothesis by manipulating cultures so that the tips of separate ureteric bud are placed on a collision course. These clustered ureteric buds prematurely ceased branching and appeared to be compressed in the direction of the collision with the neighbouring clustered kidney. This supports the idea that the tips of the ureteric bud interact with each other to avoid colliding. I did not find convincing evidence to suggest that the tips at the periphery of the colliding ureteric buds were influencing the position of their nearest neighbour. In addition to this, I attempted to compare the closest distance between tips when kidneys are placed on a collision course or are cultured in isolation.

The data presented in this thesis provide evidence both to partially support, and also to limit, the specific self-organization hypothesis tested.

Chapter 1

Introduction

This introduction will begin with an overview of kidney development followed by a description of the implications of abnormal kidney development. The process of kidney branching morphogenesis will be focused on specifically and I will outline the various models of branching from human and mouse studies. The modes of studying metanephric branching morphogenesis will be introduced before a number of key molecules required for kidney development are described. Finally, the theory concerning the intrinsic mechanisms of ureteric bud branching will be described, after which the experiments of this thesis will be briefly outlined.

1.1 Overview of kidney development

During mammalian embryogenesis, a succession of three kidneys develop; the pronephros, mesonephros and the metanephros, all of which are derived from the intermediate mesoderm. The pronephroi are the first kidneys to develop. Forming from the cranial region of the intermediate mesoderm, these tubules are often characterized as having an external glomerulus or glomus (Vize 2003). The glomus deposits its filtrate into the nearby coelomic cavity with which the pronephric tubules communicate. In amniotes the pronephros is non-functional, although in lower vertebrates that have free-swimming larvae, including amphibians and teleost fish, it develops to functional maturity (Vize 2003). The pronephroi are often unbranched in teleost fish although more primitive fish species have branched pronephroi (Vize *et al.* 2003). The pronephroi drain into the Wolffian duct. Named after the 18th century physician Kaspar Friedrich Wolff (1733–1794), the Wolffian duct runs the anterior-posterior length of the embryo in the intermediate mesoderm and terminates at the cloaca.

The mesonephric tubules develop next under the influence of the Wolffian duct (Boyden 1927; Gruenwald 1937; Waddington 1938; Sainio 2003b). The mesonephros consists of a cord of intermediate mesoderm that differentiates into numerous mesonephric tubules in a cranial to caudal sequence. Each tubule individually connects to the Wolffian duct. The mesonephric tubules do not elongate to form loops of Henle or juxtaglomerular apparatus and they contain internal glomeruli. The mesonephroi are the permanent adult kidney of fish and amphibians, but in amniotes

the mesonephric tubules are semi-functional and regress during development (Vize 2003).

As the mesonephric tubules degenerate in the amniote embryo, the final and permanent kidney, the metanephros, begins to form. In the mouse, metanephric kidney development begins at E10.5 which corresponds to week 5 of human gestation (Potter 1972; Dickinson *et al.* 2005). At this stage a simple, unbranched epithelial tube called the ureteric bud arises as a lateral outgrowth from the Wolffian duct at the level of the developing hindlimb. The ureteric bud extends outwards and invades a distinct region of the intermediate mesoderm called the metanephric blastema. The metanephric blastema at this early stage consists of undifferentiated mesenchymal cells which die unless they receive an inductive signal (Grobstein 1955; Grobstein 1967). The transcription factor *Eya1* is required for specification of the metanephric mesenchyme within the intermediate mesoderm (Sajithlal *et al.* 2005). The ureteric bud undergoes branching morphogenesis within the metanephric blastema and it forms a well-branched epithelial tree. The tips of the epithelial tree give inductive signals to the surrounding mesenchyme. The inductive signal (or signals) causes the differentiation of the mesenchymal cells into nephrons (the functional units of the kidney) or renal stroma (Sariola *et al.* 1988; Sainio *et al.* 1994; Sariola *et al.* 2003).
At E11.5 the ureteric bud has invaded the metanephric blastema and has branched dichotomously to form a T-shaped bud. It continues to ramify throughout the mesenchyme and undergoes sequential rounds of branching and elongation. When branching morphogenesis ceases, elongation of the bud continues (see the following section on branching morphogenesis). Overall the branching bud extends centrifugally throughout the mesenchyme to the periphery causing the induction of the surrounding mesenchyme along the way. Each induced nephron joins to a collecting duct via a connecting duct.

The urinary tract develops from the ureteric bud and also the urogenital sinus. The epithelium of the collecting ducts form from the ureteric bud as does the transitional epithelium (urothelium) of the renal calyces, the ureter, and trigone of the bladder. In the male the epididymis is formed from mesonephric tubules while the Wolffian duct contributes in forming the vas deferens and ejaculatory duct. The Wolffian duct essentially disappears in the female but for a few non-functional

remnants including the epoöphoron, paroöphoron and the duct of Gartner. The bladder (apart from the trigone) and the urethra develop from the urogenital sinus (endodermal derivative) (reviewed by Moore *et al.* 1998; Hynes *et al.* 2004).

1.2 Kidney development and its implications in renal disease

The kidneys are vital organs and many pathological conditions are associated with abnormalities of the renal system. During mammalian embryogenesis the foetus is highly reliant on the placenta to carry out excretion. With foeto-maternal circulation established, the foetus does not depend on its maturing kidneys to maintain homeostasis *in utero*. Consequently, specific defects in renal development quite often do not manifest themselves until the baby is born. Renal malformations arise due to disruption of the developmental program of the kidney and are classified as follows (Woolf 1997);

- Renal agenesis refers to the complete absence of the kidney and can be unilateral or bilateral.
- Dysgenesis defines a kidney composed of undifferentiated cells or metaplastic cells and a dysgenic kidney can be either small in size (aplastic) or occupy an abnormal amount of the abdominal space (as occurs with multicystic dysplastic kidneys).
- Hypoplastic or hyperplastic kidneys have respectively fewer or higher nephron numbers than normal.
- Malformations of the lower urinary tract can also arise including agenesis, upper and lower urinary obstructions (leading to hydronephros) and also vesicoureteric reflux .

This plethora of malformations can arise as a consequence of exposure to teratogens (for example administration of large doses of retinoic acid can cause agenesis (Gilbert 2002)) or due to physical obstruction of the lower urinary tract at various levels (Peters 2001). A number of syndromes also present themselves with associated renal defects. These syndromes often occur due to a disruption of a key gene involved in renal development, highlighting further how abnormal development can be implicated in renal disease. For example brachio-oto-renal syndrome is associated

with a disruption in *SIX1* (Abdelhak *et al.* 1997; Ruf *et al.* 2004), renal-coloboma syndrome associated with *PAX2* (Favor *et al.* 1996; Porteous *et al.* 2000) and Apert syndrome with *FGFR2* (Wilkie *et al.* 1995).

It is suggested that there is a link between nephron endowment in the kidney and the development of hypertension (Cullen-McEwen *et al.* 2003). As nephrogenesis finishes in humans during week 34 of gestation the endowment of nephrons is highly dependent on the correct regulation of renal development. Nephrogenesis is dependent on three factors (Clark *et al.* 1999):

- Optimal branching of the ureteric bud.
- Optimal induction of the surrounding mesenchyme by the tips to form condensates.
- Efficient conversion of these condensates to epithelial renal vesicles and maturation of these vesicles into the various cell types of a nephron.

Therefore deregulated branching morphogenesis has clear implications for disease in later life. It is logical to assume that the development of the metanephric kidney relies on a regulated epithelial branching program to achieve an optimally branched architecture. Abnormal branching processes can compromise the functionality of kidneys in the neonate.

1.3 Overview of branching morphogenesis

Branching epithelia can arise from any of the three germ layers, the endoderm (lung, pancreas, salivary, prostate) mesoderm (kidney) or the ectoderm (mammary glands). The branching epithelium of the kidney is unlike many of the other branching epithelia, such as the pulmonary epithelium or the mammary epithelium, which essentially always remain a closed epithelial system. The situation with the ureteric bud is a little more complicated as each nephron tubule must join up, via connecting ducts, to the branched epithelium. In this way a uriniferous tubule, completely patent from the urinary space of the Bowman's capsule through to the renal papilla, is formed.

In general, epithelial branching is driven in two main ways. Either there is a rearrangement of the cells of the branching epithelium to produce new branches or there is a differential increase in cell proliferation. The trachea of *Drosophila* uses the former method to produce branches. In this case, cells of the epithelium elongate and ramify without a concomitant increase in cell proliferation (Beitel *et al.* 2000). In vertebrate organs such as the lung (Goldin *et al.* 1984) and ureteric bud (Michael *et al.* 2004), cell proliferation is used to give rise to new branches although cell rearrangement may take place as well.

Branching patterns can arise in two ways during organogenesis (figure 1.1). The two principal modes of branching are termed terminal (dipodial/tripodial *etc.*, figure 1.1b) or lateral (monopodial, figure 1.1a) (al-Awqati *et al.* 1998). Terminal branching events occur when new branches form from the end regions (*i.e.* tips) of the branching epithelium. Terminal branches can arise in a bifid (dipodial), trifid (tripodial) or even a carrefours (division into 4 daughter branches) manner. However it is assumed that trifid and carrefours branching is seen when multiple rounds of bifid branching occur in rapid succession with little time allowed for elongation or remodeling of the intervening stalk (al-Awqati *et al.* 1998). Terminal branching means that new branches arise from pre-existing tips.

In contrast, lateral (monopodial) branches arise from the sides of the stalk regions. Branches can be spaced at regular or irregular intervals. In the case of lateral branching events, new branches do not form from pre-existing tips at the ends of the epithelium. It is thought that both lateral and terminal branching occurs to some extent during the development of all branched organs although lateral branching is the predominant mode of branching seen during lung development (Lin *et al.* 2003) and terminal branching is seen most frequently during metanephric kidney development (al-Awqati *et al.* 1998; Watanabe *et al.* 2004).

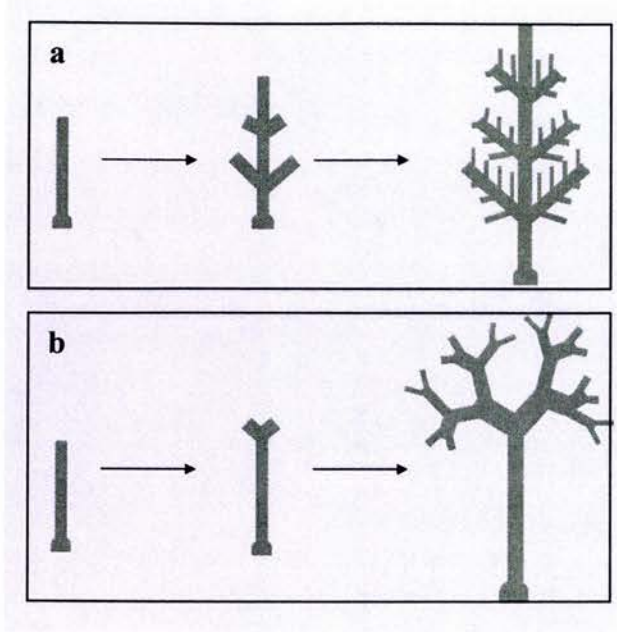


Figure 1.1: Branching patterns

Epithelial morphogenesis through branching can give rise to quite different patterns of branches. During organogenesis, branches can arise predominantly as lateral branches from the stalks of the epithelium (monopodial branching) (a) or can arise from the terminal ends of the epithelium (dipodial/tripodial *etc.*) (b).

1.4 Modes of branching in the metanephric kidney

1.4.1 Human studies

Original investigations into branching morphogenesis of the kidney involved analysis of dissected human foetal kidneys at various developmental stages (Peter 1909; Oliver 1968; Potter 1972). Oliver preformed very fine dissections and camera lucida tracings of isolated collecting ducts and their associated nephrons and from these dissections he infers a model of human renal branching morphogenesis (Oliver 1968). Peter dissected kidneys from embryos as early as 5 months old while the earliest human kidney microdissected by Oliver and Potter were from a 2⅛th month and 2 month old foetus respectively (Oliver 1968; Potter 1972).

Oliver's studies make inferences about kidney development from the stage when the renal calyces are forming and the initial branching events of the ureteric bud have already occurred. Oliver used Roman numerals to refer to the successive generations of branches and I will do likewise. Overall it seems that XV generations of branches form in total during human kidney development (Oliver 1968). Potter suggests the first VI to the first X generations of branches remodel to form the renal

pelvis and calyces of the adult kidney, while Oliver proposes the first V generations contribute to these structures (Oliver 1968; Potter 1972). Sometimes, the early branches advance forward so quickly that nephrons may not get induced at each division (Potter 1972). It is also postulated that the nephrons that are induced to form during early branch divisions may not advance with the duct and degenerate similarly to the degeneration of the pronephric and mesonephric tubules (Oliver 1968; Potter 1972). Evidence of such degenerations has been found in histological sections of human embryos (Potter 1972). Division in the human kidney is mostly dichotomous but at earlier branching stages trifid and even carrefour branching from the tips are evident (Oliver 1968). However, trifid or carrefour branch points can be remodelled to give the appearance of dichotomy of the collecting duct (Oliver 1968). After three months of gestation only dichotomous divisions occur (Oliver 1968). Oliver categorizes two periods of development based on nephron induction and associated collecting duct growth. They are termed the closed divided system where duct division and nephron induction are actively occurring and the open direct system where division has ceased but nephron induction continues (Oliver 1968).

1.4.1.1The closed divided system of kidney development (figure 1.2)

According to Oliver, the initial collecting ducts that emerge from the renal papilla represent at least the VIth generation of branches and the nephrons induced by the earlier generations do not develop into fully mature nephrons, but die (Oliver 1968). Therefore nephrogenesis begins with the VIth branches. After VI generations of branching, divisions are dichotomous (although previous branching events may be trichotomous or carrefour). The ureteric bud tip of each ampulla divides dichotomously to give rise to two closely associated sister tips. Before advancing, each of these two sister tips induces the formation of one nephron in the mesenchyme occupying the lateral side of each tip (*i.e.* not within the space of the cleavage furrow). Each nephron attaches to its tip via a connecting tubule and the tips advance through the mesenchyme. The nephron remains attached to the advancing tip. Each tip then branches dichotomously again to produce two sister tips, one of which has an attached nephron from the previous branching event while the other has

no attached nephron. Each tip induces the formation of a new nephron which attaches to the ampulla before the tip advances forward again. Dichotomous branching proceeds in this manner until the XVth generation of branches have formed (this lasts until the fourth month of human gestation). Nephrons advance with the tip towards the periphery of the organ. Arcades of nephrons may form when the connecting ducts of the nephrons join together to attach just behind the tip by means of a common connecting duct. This period, in which branching and nephron induction alternate with each other, is the closed divided system of metanephric kidney development. It is followed by the next period of growth, the open direct system.

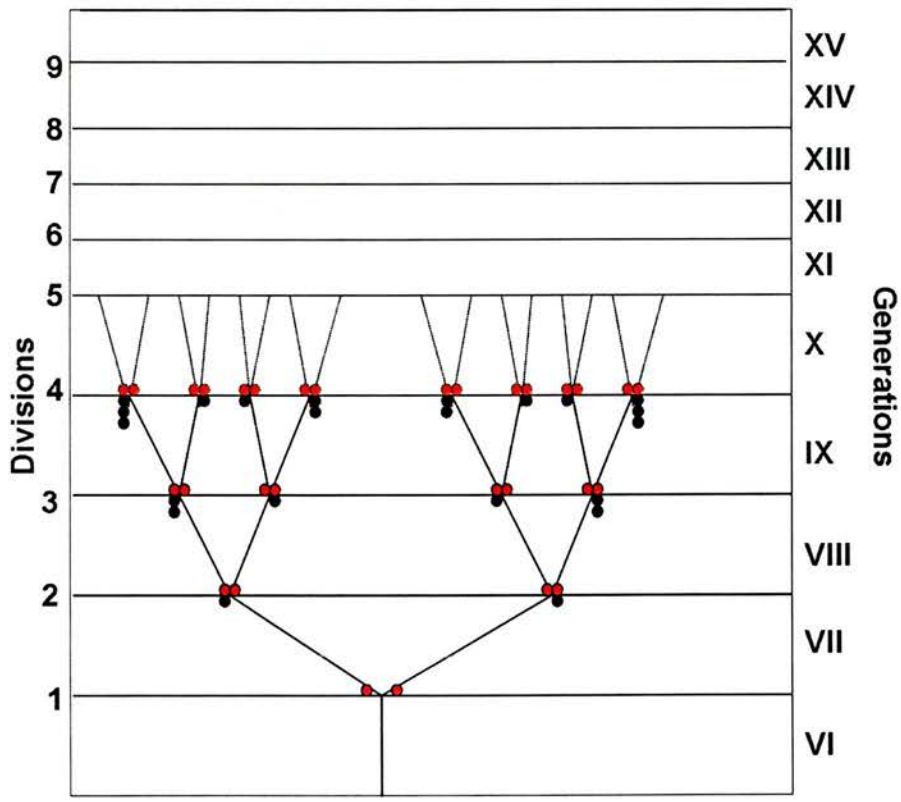


Figure 1.2: The closed divided system of metanephric kidney development. Adapted from Oliver 1968.

Each tip of a newly formed ureteric bud branch induces one new nephron (red circle) after every division. Nephrons from previous inductive events (black circles) remain attached to the advancing ampulla. Subsequently, aggregates of nephrons can form on the tips. These are known as nephron arcades if they are attached via a common connecting duct.

1.4.1.2 The open direct system of kidney development

The open direct system is defined as the period in which the tips continue to advance towards the periphery of the kidney but they no longer branch. Nephrons are induced at intervals and connect, via their individual connecting ducts, directly to the collecting duct, thus forming a cortical lateral system of nephrons (figure 1.3). When nephrogenesis is complete (in the human this is around E238 and in the mouse it is around postnatal day 5-7 (Dickinson *et al.* 2005)), the tips of the collecting duct terminate by merging with the connecting ducts of two adjacent nephrons.

Both branching of the collecting ducts and nephron induction is fully completed by the end of the open divided system (Oliver 1968). When the open divided period finishes there follows a period of maturation and elongation of the collecting ducts and nephrons (Oliver 1968).

Oliver describes all branching events during the closed divided system as being dichotomous. As mentioned, earlier branching events often appear as trifurcations and carrefour divisions from the ampullae (Oliver 1968). These divisions are at termini of the ureteric buds (the tip). Oliver does not suggest that lateral branch formation occurs during ureteric bud branching (Oliver 1968). It is possible that lateral branching events could have been overlooked in this study especially as lateral branches can appear similar to, and can be mistakenly identified as, terminal branches quite soon after they have arisen.

Hypothetically, according to the closed divided system the final 9 divisions of the branched ureteric bud tree would result in increasing number of tips in the sequence: 2, 4, 8, 16, 32,...512, or 2^n where n = the number of generations of branches. As each tip produces one new nephron the number of nephrons for each generation of branching would be 2, 6, 14, 30,...1022 or $2(2^n)$-2 (Oliver 1968). At the end of the closed divided system the number of nephrons per papillary collecting duct is predicted to be 1022. Oliver measures the average number of papillary collecting ducts as 44 with 8 papillae in the human kidney. Therefore during the closed divided system potentially 359,744 nephrons are formed. Oliver calculated that on average 767,819 nephrons are induced during the open direct system, giving a total of 1,127,563 nephrons in the human kidney. For comparison, in the mouse there are

about 8,000 nephrons in a newborn kidney (Cebrián *et al.* 2004) and about 11,000 when mature at P30 (Dickinson *et al.* 2005).

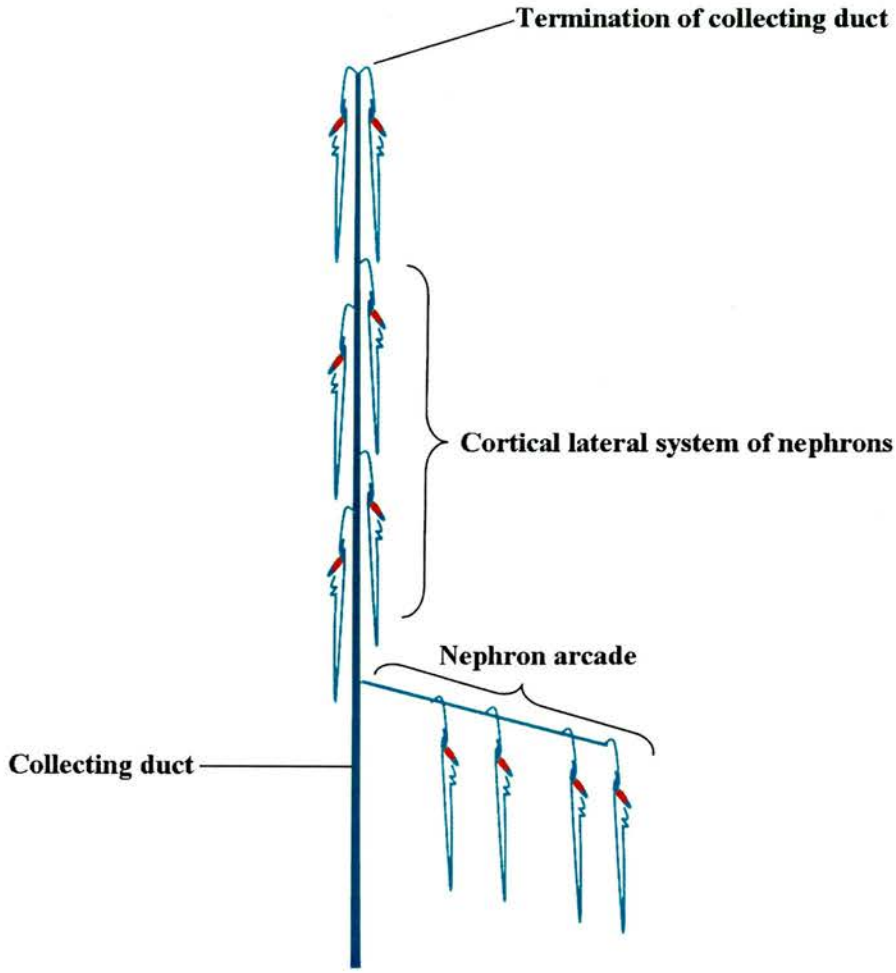


Figure 1.3: The arrangement of nephrons with the collecting duct system.

The closed divided system of kidney development accounts for the formation of nephron arcades. With nephron arcades collections of nephrons attach via a common connecting duct to the collecting duct. Cortical lateral systems of nephrons form during the open direct system of kidney development. The collecting duct does not divide during this period and nephrons attach via individual connecting pieces to the collecting duct. When nephrogenesis is complete the collecting duct tip terminates by merging with the connecting duct of two adjacent nephrons.

From his dissections and inferences made on the works of Peter (Peter 1909), Oliver explains how nephrons are absent from the lower levels of the ureteric bud and all are all located in the cortical region at the periphery of the kidney (Oliver 1968). As

nephrons are always found to attach to the ampulla of the collecting duct tip, it is suggested, originally by Peter, that these nephrons are carried to the cortical region of the kidney by differential growth of the collecting duct behind the area of nephron attachment (Oliver 1968). If this zone of proliferation was positioned between the very end of the collecting duct and the attachment of the nephron, the nephron would not advance with the tip and would attain positions of attachment at various levels throughout the collecting duct system (Oliver 1968). As the proximal and distal portions of nephrons are always located in the cortical region of the kidney, there must be differential proliferation behind their site of attachment to carry them forward to the periphery of the organ along with the advancing tip regions of the collecting duct (Oliver 1968). Although Peter proposes this mechanism of growth, he is unable to provide evidence of it (Oliver 1968). Oliver, though his dissections of collecting ducts, simply shows that areas of proliferating cells (as seen by cell dense areas on photographs) are often seen in the tip regions, around the attachment sights of the developing nephrons (Oliver 1968).

Similar dissection studies by Potter create a different view of kidney morphometrics during development (Potter 1972). Oliver suggests that each papilla has 44 papillary ducts and that the papilla is formed from the 5 initial generations of ureteric bud branches. However if divisions are mostly dichotomous, more than 5 generations of branches must give rise to the papillae. Potter addresses this discrepancy by proposing that the initial 3-5 generations of branches produce the renal pelvis and that subsequent 3-5 divisions give rise to the calyces and papilla (Potter 1972). She also states that the average number of papillary ducts lies between 10 and 25 (Potter 1972). After the formation of the papilla an additional 7-8 divisions are generated (Potter 1972). Potter holds the view that there are four periods of growth during human kidney morphogenesis (Potter 1972):

1.4.1.3 Period 1

This period last from the initial outgrowth of the ureteric bud until week 14-15 (Potter 1972). During this period branching proceeds principally in a dichotomous manner (Potter 1972). Each tip induces the formation of a new nephron only if it does not have a nephron attached (Potter 1972). Therefore, a tip ampulla can produce

a new tip during each round of branching morphogenesis but a tip can only induce a new nephron if it has not got one attached already (Potter 1972). (This disagrees with Oliver's finding that tips can accumulate on the tip ampulla during this early stage of development). In this way, nephrons attached to the various tips are all of differing stages of development. Potter suggests that the differing ages of these nephrons can easily be demonstrated by microdissection and examination of the length of the loop of Henle (Potter 1972).

1.4.1.4 Period 2

Period 2 is defined by the induction of nephrons about the collecting duct tip even though a nephron is already attached and the cessation of collecting duct branching (Potter 1972). It lasts from 14-15 to 20-22 weeks and nephron arcades form during this period (Potter 1972). The tips induce nephrons in rapid succession and these nephrons attach via common connecting ducts to the tip ampulla (Potter 1972). Overall Potter argues that there is a definite interval between the cessation of tubular division and the time when nephrons attach directly as cortical lateral nephrons. It is during this interval that nephron arcades form (Potter 1972). Higher order arcades should be evident if Oliver's view of arcade formation is correct but Potter suggests there is little evidence of this (Potter 1972).

1.4.1.5 Period 3

This period lasts until week 32-36 (Potter 1972). During period 3 the tips advance past the point of arcade attachment (Potter 1972). They seldom branch can induce nephron formation only when there is none already attached (Potter 1972). The new nephrons that are induced are attached behind the zone of proliferation in the tip (Potter 1972). In this manner, cortical lateral systems of nephrons form.

1.4.1.6 Period 4

This period is a period of maturation and it continues into adult life (Potter 1972). The tip ampullae disappear by inducing terminal nephrons, after which branching and nephron induction can not occur (Potter 1972). All changes during this time are a result of interstitial growth and cellular differentiation (Potter 1972).

al-Awqati and Goldberg present a review of Oliver's work (al-Awqati *et al.* 1998). They describe the terminal bifid system of branching whereby terminal branching and lateral branching alternate (al-Awqati *et al.* 1998). This system of branching is based on the assumption that once a tip becomes attached to a nephron it can no longer divide (al-Awqati *et al.* 1998). However, neither Potter nor Oliver suggest lateral branching takes place during human kidney development. They do not suggest that tips are incapable of branching when a nephron is attached. Therefore there seems to be some ambiguity concerning al-Awqati and Goldberg's view of branching morphogenesis. (al-Awqati *et al.* 1998).

Potter describes arcade formation as a separate stage in collecting duct development after collecting duct branching ceases, whereas Oliver views it as an accumulation of nephrons that form during branching of the collecting duct system. Although both Potter's and Oliver's views conflict in regard to human kidney development, both account for the arrangements between nephrons and collecting ducts in the adult cortex. It is clear that the tips of the ureteric bud are the areas where active branching morphogenesis and nephron induction take place. Branching takes place during early kidney development, after which nephron formation continues to occur.

1.4.2 Mouse studies

A recent histological examination and quantification of various parameters during murine kidney development was carried out. It comprises a compilation of morphometrics such as surface area, tip number, glomeruli number, branching events *etc.* for kidneys from E11.5 until the newborn stage (Cebrián *et al.* 2004). It is estimated that 10-11 branching events take place during mouse kidney development (Cebrián *et al.* 2004). The authors note a number of points of interest. Firstly it does not seem that the number of branches correlate with the number of induced nephrons. In fact it appears that up until E16.5 only 70% of the generated ureteric bud tips have induced a nephron (Cebrián *et al.* 2004). From E16.5 until birth there is a 10-fold increase in the number of nephrons, while tip number only increases 2.7 fold (Cebrián *et al.* 2004). This would suggest that there is an early period of

development when the rate of branching of the collecting duct is higher than the rate of nephrogenesis. This could be similar to period 1 of human renal development as proposed by Potter or similar to Oliver's closed divided system of branching. Although both Oliver and Potter seem to suggest that nephron number is dependent on the number of tips, both recognise that the early branches may not give rise to fully functional nephrons (Oliver 1968; Potter 1972) or that branches may not always induce a nephron, especially when branching events occur in rapid succession (Potter 1972). There is no evidence of nephron apoptosis in the mouse kidney (Cebrián *et al.* 2004). The Cebrián *et al.* study measures nephron number by counting glomeruli. It is possible that by counting nephrons in this way there is an underestimation of nephron numbers as early nephrons would be overlooked at each developmental stage analysed (Cebrián *et al.* 2004).

Following this early period the rate of branching morphogenesis slows down and there is a huge increase in nephrogenesis (from E16.5 onwards) (Cebrián *et al.* 2004). Perhaps this is analogous to Potter's period 2/3 of human kidney development or to Oliver's open direct system. It is unclear whether lateral branching occurs during mouse kidney development *in vivo*. For 10 rounds of branching in the mouse kidney, as proposed by Cebrián *et al.* there is a final number of 1597±201.2 tips (Cebrián *et al.* 2004). The number of tips expected to form if branching was terminal and dichotomous (2^{10}) is 1552.

Also recognised is the interstitial increase in length of the outer-medullary collecting ducts. This begins about E15.5 and correlates well with the onset of stromal differentiation of the cells in between the ureteric bud stalks (Cebrián *et al.* 2004). Cebrián *et al.* describes two axes of mouse renal growth. During development there is an expansion in volume of the cortical region and the medulla. The growth of the cortex is circumferential while it is longitudinal in the medulla (figure 1.4).

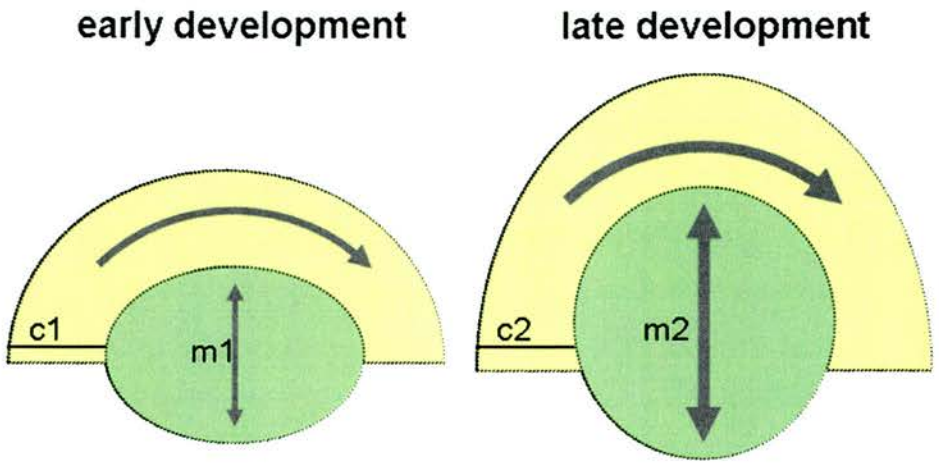


Figure 1.4: 2D representation of growth of the mouse kidney. Adapted from Cebrián *et al.* 2004.

The medulla (green) grows longitudinally over time (m1 < m2) while the cortex (yellow) grows circumferentially. The cortex increases its volume without changing its thickness (c1 = c2).

Other investigators propose that murine collecting duct morphogenesis can be divided into 4 stages (figure 1.5) (Stuart *et al.* 2003; Nigam 2003; Meyer *et al.* 2004; Sampogna *et al.* 2004; Bush *et al.* 2004; Shah *et al.* 2004; Steer *et al.* 2004). The initial outgrowth of the ureteric bud is stage 1. This is followed by early rounds of branching morphogenesis (Stage II), late branching (Stage III) and stage IV, when branching ceases and terminal differentiation occurs. A fifth stage may be operative which does not involve branching but is concerned with repair and maintenance of the collecting duct system. When exactly the transition between the stages 2-4 occurs during kidney development is not clear.

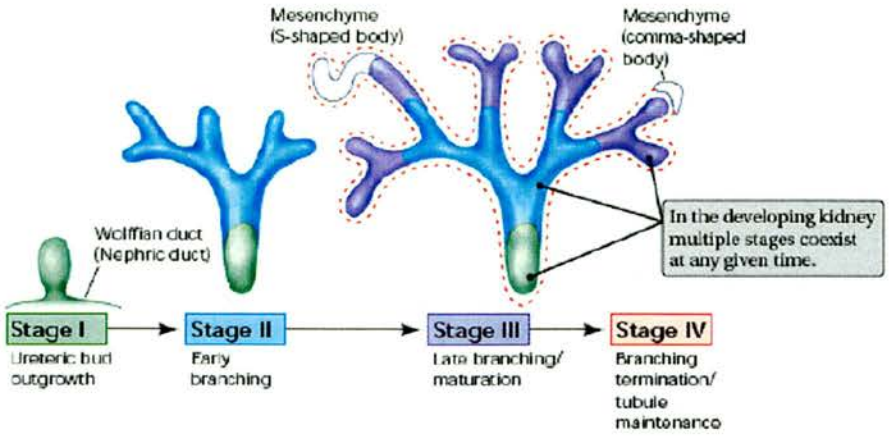


Figure 1.5: The four stages of collecting duct development. Taken from Sampogna *et al.* 2004.

Although both mouse and human studies of dissected or sectioned kidneys have provided great insight into branching morphogenesis, these studies are often exhaustive and technically difficult to analyse. It is hard to interpret simply the mechanisms of branching when dealing with the ever increasing complexity of the kidney over time.

Mouse studies of metanephric branching morphogenesis have focused on the analysis of organ development *in vivo* and in organ culture. Particularly insightful were studies carried out using time lapse imaging of *Hoxb7*-GFP kidneys in organ culture (Watanabe *et al.* 2004). From these studies it has been shown that the principle type of branching morphogenesis, seen during *in vitro* mouse kidney development, is terminal branching although lateral branching also occurs to a lesser extent (Watanabe *et al.* 2004). Using time lapse imaging of *ex vivo* cultured transgenic kidneys, in which the ureteric bud was expressing EGFP under the *Hoxb7* promoter, Watanabe *et al.* demonstrated that lateral branching events account for 6% of all branching events and that these lateral branches almost always arise from the 2nd and 3rd generations of branch segments. Lateral branches are not programmed to continue to branch laterally and quite often the tips of lateral branches can subsequently undergo terminal branching events (Watanabe *et al.* 2004). Terminal branching accounts for the rest of the branches that form with 20% of these being

trifurcations and 80% being bifurcations (Watanabe *et al.* 2004). The branching pattern of the ureteric bud is not completely predictable and there is some variation in the branch patterns formed by kidneys in organ culture. However the pattern formation is not random either and similarities between all kidneys are seen (Watanabe *et al.* 2004). The initial branching event of the ureteric bud gives rise to the 2nd generation of branches through a bifid branching event and very often the 3rd branch generations form from a trifid branching event (Watanabe *et al.* 2004). Also, the time lapse studies on *Hoxb7*-GFP expressing ureteric buds suggest that the alternation of lateral and terminal branching, as suggested by al-Awqati and Goldberg (al-Awqati *et al.* 1998), does not occur at least for mouse kidneys *in vitro* (Watanabe *et al.* 2004).

Molecular analysis using BrdU proliferation assays on organ cultured mouse kidneys show that proliferation is comparatively higher in the tips of the ureteric bud compared to the stalks (Michael *et al.* 2004). Although this analysis was not performed in relation to nephron attachment, it correlates well with the observations and hypothesis of Oliver and Peter (Peter 1909; Oliver 1968) that were discussed earlier. This also confirms the idea that branching morphogenesis of the ureteric bud is driven by localised cell proliferation (Michael *et al.* 2004).

1.5 Models of metanephric branching morphogenesis

There are various ways to study branching morphogenesis of the ureteric bud. Grobstein pioneered the organ culture technique for metanephric kidneys (from as early as E10.5) (Grobstein 1953; Grobstein 1956; Grobstein 1957; Saxen 1987; Sainio 2003a). Kidneys cultured in organ culture continue to grow and develop in a manner highly reflective of *in vivo* kidney development with multiple rounds of branching and nephrogenesis occurring. These kidneys are highly manipulable as many morphogens, inhibitors, siRNA *etc.* can be added to the culture medium. Molecular and histological analysis can be performed on organ cultured kidneys.

The mesenchymal component and the epithelial component of the metanephric kidney can be separated and cultured independently. The mesenchyme can be

induced in transfilter culture with either the native inducer, the ureteric bud, or with heterologous inducers such as the dorsal spinal cord (Saxen 1987).

Similarly, the ureteric bud can be cultured independent of its surrounding mesenchyme. If cultured alone without any support matrix, the ureteric bud flattens out, sheds epithelial cells and within 2 days has virtually disappeared (Grobstein 1955; Saxen 1987; Sainio 2003a). However the bud grows well in combination with heterologous mesenchyme such as lung mesenchyme (Kispert *et al.* 1996; Sainio *et al.* 1997; Lin *et al.* 2001) but in a pattern more reminiscent of lung branching morphogenesis (Lin *et al.* 2001).

Isolated ureteric bud culture can also be achieved by culturing the naked bud in an artificial matrix with exogenous growth factors:

- Buds grow well in a 1:1 collagen type I, matrigel matrix using conditioned medium from the BSN cell line (metanephric mesenchymal immortalized cell line) and GDNF (Qiao *et al.* 1999a).
- 1:1 collagen type I, matrigel matrix also supports ureteric bud branching when GDNF, FGF1 and pleiotrophin are added to normal medium (Sakurai *et al.* 2001). As a modification of these two methods buds can be grown in 1:1 collagen type I, matrigel matrix with the addition of GDNF, FGF1 in BSN conditioned medium (Meyer *et al.* 2004).
- Naked ureteric buds can also be grown in Matrigel in complete medium supplemented with GDNF, HGF and FGF-7 (Karihaloo *et al.* 2001).

Certain cells of the mature collecting duct have also been found to branch well when embedded in matrices and supplemented with extra growth factors. One such cell line is mIMCD$_3$. Derived from the inner medullary collecting duct of the mouse these cells branch well when embedded in collagen type I or a mixture of matrigel and collagen type I and cultured using HGF supplemented medium (Cantley *et al.* 1994). Another cell line MDCK cells from an unknown region of the kidney of an adult dog (Feifel *et al.* 1997) also branch well in collagen gels in the presence HGF (Montesano *et al.* 1991). An immortalised cell line derived form the ureteric bud can induce nephrogenesis (Barasch *et al.* 1996) and is also capable of undergoing branching morphogenesis in a collagen I matrigel matrix supported by BSN medium

(Sakurai *et al.* 1997). These cultures of cells provide a means of analyzing the responses of branching cell types without having to consider the indirect effects caused by mesenchymal development as sometimes occur in organotypic culture. It has been suggested however that the mechanism used by these cell models is somewhat different to that of the isolated ureteric bud (Meyer *et al.* 2004).

Transgenic studies have also been a powerful tool to study branching morphogenesis. A plethora of genes crucial for regulated branching have been found through the generation of knock out mice or through the identification of mutations linked to known syndromes. Often studies of dissected and cultured kidneys result in the analysis of static images which although very informative can be limited in shedding light on the dynamic process of branching morphogenesis. Transgenic tools have helped overcome these limitations to some degree. For example, studies using *hoxb7*-GFP mice along with time lapse imaging methods (Watanabe *et al.* 2004) have provided clearer insight into branching morphogenesis as a continuous process rather than 'snapshot' information.

Overall the methods of studying branching morphogenesis are varied and when used in conjunction with each other and with molecular biological techniques, help to unravel the highly complicated process of epithelial branching.

1.6 Regulation of kidney development

The ureteric bud and metanephric mesenchyme reciprocally interact with each other during kidney development. Understanding the molecular basis of such complex interactions is quite important and numerous key morphogens, transcription factors and receptors have been identified as being crucial to the process. Two ideas about the control of branching morphogenesis are held. The mesenchyme itself is thought to communicate to the ureteric bud via spatially and temporally regulated signals and thereby induces the ureteric bud to branch in a regulated manner. The second idea focuses on an intrinsic ability of the ureteric bud to regulate its own pattern of branching morphogenesis. The idea of an intrinsic branching program for the ureteric bud is based on the fact that mesenchyme independent culture of the ureteric bud in a 3D matrix seems to be controlled and does not appear to be a

completely random process (Qiao *et al.* 1999a; Karihaloo *et al.* 2001; Meyer *et al.* 2004). These two ideas of how branching morphogenesis is controlled in the ureteric bud may not be independent mechanisms but may depend on each other to correctly shape the overall architecture of the ureteric bud epithelium.

Some of the major molecular players and their roles in kidney development are briefly outlined below (reviewed in Davies *et al.* 2002; Davies 2002). A more comprehensive list can be found at the Kidney Development database (Davies 2005). Figure 1.6 highlights some of the important molecules involved in ureteric bud branching morphogenesis (Davies *et al.* 2002).

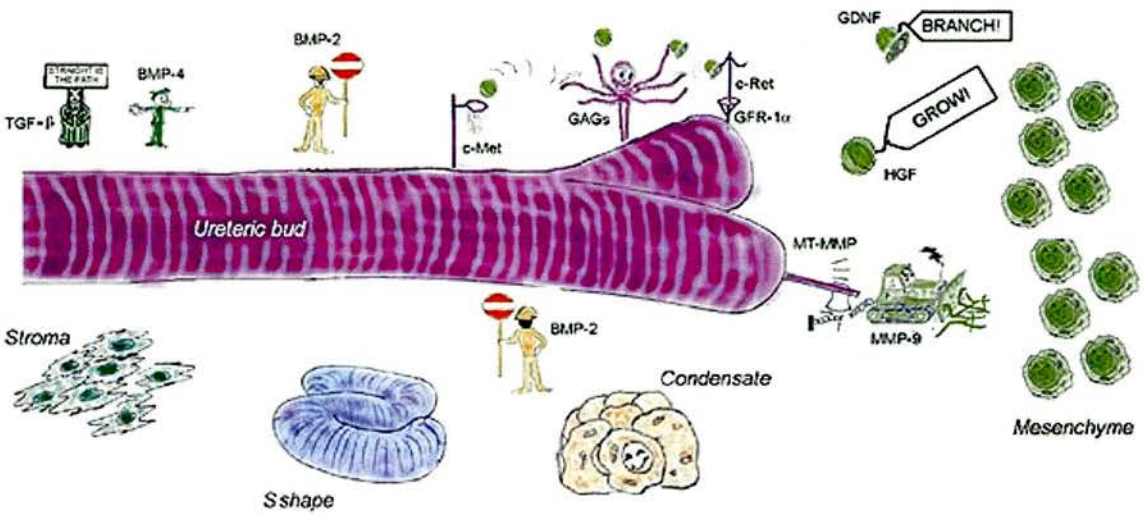


Figure 1.6: Molecules involved in regulating kidney development. This cartoon highlights some of the important molecules involved in regulating kidney development (Davies *et al.* 2002)

1.6.1 Morphogens

1.6.1.1 Glial cell derived neurotrophic factor (*Gdnf*)

The GDNF family of signalling molecules include artemin, neurturin, persephin and GDNF itself (Airaksinen *et al.* 2002). They are members of the TGF beta superfamily although they do not signal through receptor serine-threonine

kinases (Airaksinen *et al.* 2002). Instead, these ligands signal through the Ret receptor tyrosine kinase and activation is facilitated by the GFRα co-receptors.

GDNF is probably the best characterized kidney development morphogen. GDNF dimerises, binds specifically to either GPI anchored or soluble GFRα1 co receptor, before interacting with RET (Jing *et al.* 1996; Treanor *et al.* 1996; Paratcha *et al.* 2001). In the developing kidney GDNF is secreted by the uninduced metanephric mesenchyme (Hellmich *et al.* 1996; Suvanto *et al.* 1997; Sainio *et al.* 1997). The *Ret* receptor is initially expressed by the ureteric bud at early stages of its outgrowth and later becomes expressed only by the tips of the ureteric bud (Sainio *et al.* 1997). The *Gfrα1* co-receptor is expressed by the ureteric bud epithelium and also by the surrounding mesenchyme (Sainio *et al.* 1997). GDNF binds to the tips of the ureteric bud (Sainio *et al.* 1997).

Both *Gdnf* (Pichel *et al.* 1996; Sanchez *et al.* 1996) and its co-receptor *Gfrα1* (Enomoto *et al.* 1998) are absolutely required for kidney development. The RET receptor, particularly the RET9 isoform, is also needed for kidney development (Schuchardt *et al.* 1994; de Graaff *et al.* 2001). As well as suffering from defects in their enteric nervous system, homozygous knockouts of *Gdnf, Ret* and *Gfrα1* present with renal defects ranging from severe kidney dysgenesis to complete agenesis due to defective outgrowth and branching of the ureteric bud. Renal agenesis is not 100% penetrant in these knockout mice. Mice heterozygous for *Gdnf* also present with kidney defects including unilateral agenesis and hypoplasia (Cullen-McEwen *et al.* 2003; Enomoto *et al.* 1998).

Nrtn (neurturin) is also expressed in the developing kidney specifically in the tips of the ureteric bud (Widenfalk *et al.* 1997) suggesting it acts as a autocrine factor. *Nrtn* knockout mice do not present with any kidney defects (Heuckeroth *et al.* 1999). Interestingly, in culture NRTN promotes branching morphogenesis but only in the presence of GDNF function blocking antibodies (Davies *et al.* 1999; Milbrandt *et al.* 1998). PSPN (persephin), also expressed by the developing kidney, supports branching morphogenesis of the ureteric bud when GDNF signalling has been diminished by sodium chlorate (which on its own is an inhibitor of ureteric bud branching) (Milbrandt *et al.* 1998).

1.6.1.2 Hepatocyte growth factor (*Hgf*)

HGF is a growth factor secreted by the metanephric mesenchyme and signals through the MET receptor which is expressed by the ureteric bud and also the mesenchyme. Knockouts for *Hgf* or *Met* do not have any kidney defects and die *in utero,* after kidney development has been initiated, due to placental and liver defects (Bladt *et al.* 1995; Schmidt *et al.* 1995). However perturbation of HGF signalling in culture using anti-HGF antibodies blocks branching morphogenesis (Woolf *et al.* 1995) and is often used in studies on both organ and cell culture models of branching morphogenesis. Regional activation of HGF is suggested to be mediated by a HGF activator which is found specifically around the tips of the ureteric bud and so may ensure a localised activation of signalling by HGF (van Adelsberg *et al.* 2001).

1.6.1.3 WNT proteins

WNT proteins comprise a large family of secreted proteins that are implicated in the development of many organs including the kidneys. They signal through frizzled receptors.

At least five *Wnt* genes are expressed in the developing kidney, *Wnt4, Wnt6, Wnt7, Wnt9* and *Wnt11 (Carroll et al. 2005). Wnt6* (Itaranta *et al.* 2002), *Wnt7, Wnt9,* (Carroll *et al.* 2005) and *Wnt11* (Christiansen *et al.* 1995; Kispert *et al.* 1996) are each expressed specifically by the ureteric bud while *Wnt4* is expressed by the developing nephrons (Stark *et al.* 1994; Kispert *et al.* 1998).

Wnt11 has a very interesting expression pattern in the developing metanephros (Christiansen *et al.* 1995; Kispert *et al.* 1996). As the ureteric bud grows outward from the mesonephric duct at E10.5, its tip expresses *Wnt11* while the cells of the stalk do not (Kispert *et al.* 1996). By E11.5, the first branching process is complete and *Wnt11* expression is now confined to the tips of the two invading daughter branches of the ureteric bud (Kispert *et al.* 1996). In kidneys from E12.5 onwards *Wnt11* expression is seen as a spotted pattern at the periphery of the kidney corresponding to the tips of the growing bud (Kispert *et al.* 1996). Its expression correlates with the site where nephron induction signals are thought to eminate

although WNT11 is not able to induce tubule formation (Kispert *et al.* 1998). *Wnt11* expression is dependent on sulphated proteoglycans (Kispert *et al.* 1996). Its expression is specifically down regulated in $Ret^{-/-}$ kidneys and $Wnt11^{-/-}$ mutants show reduced *Gdnf* expression in the mesenchyme although *Ret* continues to be expressed (Majumdar *et al.* 2003). These findings suggest that WNT11 cooperates with RET/GDNF signalling in a feedback loop to coordinate branching of the ureteric bud (Majumdar *et al.* 2003). In the absence of *Wnt11,* branching morphogenesis is abnormal, resulting in kidney hypoplasia although some branching does occur (Majumdar *et al.* 2003).

Wnt7 has a similar expression as *Wnt9* in the developing kidney (Carroll *et al.* 2005). Both are expressed preferentially by the stalk of the ureteric bud, although *Wnt7* is solely expressed by the extrarenal ureter while *Wnt9* expression extends further into newly forming (distal) stalk regions (Carroll *et al.* 2005). *Wnt9* knockout mice have only vestigial kidneys composed of a few ureteric bud branches but no nephrons (Carroll *et al.* 2005). It is possible that the branching defects observed in these kidneys are an indirect effect of defective nephrogenesis (Carroll *et al.* 2005).

Wnt4, which is essential for kidney development, is expressed by the condensing metanephric mesenchyme (Stark *et al.* 1994). This protein is thought to act to maintain the forming nephrons (Kispert *et al.* 1998) and its up regulation is dependent on *Wnt9* (Carroll *et al.* 2005).

WNT4, WNT9 and WNT7 can induce nephrons in isolated mesenchyme but WNT11 does not have this ability (Kispert *et al.* 1998; Carroll *et al.* 2005). WNT6 can induce nephrons even in $Wnt4^{-/-}$ mesenchyme and is expressed more strongly in the tips of the ureteric bud than in the stalk suggesting it is a potential signal for nephron induction (Itaranta *et al.* 2002). WNT1 which is expressed by the dorsal spinal cord can induce nephrogenesis, although it is not expressed by the kidney itself (Herzlinger *et al.* 1994).

1.6.1.4 Transforming growth factor β (*Tgfβ*) signalling molecules

The signalling molecules belonging to this family all signal via type I and type II serine-threonine kinases. Signal transduction leads to the phosphorylation and

subsequent activation of the SMAD family of transcription factors (Piscione *et al.* 1997; Oxburgh *et al.* 2004).

Tfgβ1 itself is expressed predominantly by the developing stroma (Partanen 1990) but there is no obvious renal defect in knockout models (Boivin *et al.* 1995). In culture, exogenous TFGβ1 inhibits branching, decreases the branch angle between branches and reduces the area of the kidney as a whole (Ritvos *et al.* 1995; Bush *et al.* 2004).

Also a member of the TGBβ family, activin is expressed by the metanephric mesenchyme and in culture it appears to negatively regulate branching morphogenesis although the bud continues to elongate (Bush *et al.* 2004; Ritvos *et al.* 1995). Its natural inhibitor follisatin is also implicated in branching morphogenesis as it rescues the defects caused by activin in cell culture models (Maeshima *et al.* 2001). Follistatin is expressed on the surface of the activin responsive cells and causes internalization of activin so that it gets degraded (Maeshima *et al.* 2001). Knockout studies which abrogate signalling by activin, through the expression of a truncated activin type II receptor, highlight a mild renal phenotype whereby there is an increase in nephron number possibly due to increase ureteric bud branching (Maeshima *et al.* 2001).

1.6.1.5 Bone morphogenetic proteins (BMP)

Another family of TGFβ signalling proteins, the BMPs have a significant role to play in kidney development. Although *Bmp2, 3, 4, 5* and *7* are expressed during kidney development in various compartments, only mice mutant for *Bmp7* show a clear renal phenotype (Godin *et al.* 1999). *Bmp7* is expressed by the entire ureteric bud and also in the condensed metanephric mesenchyme. It is absolutely necessary for kidney development. *Bmp7* knockout mice die soon after birth due to completely penetrant severe bilateral renal dysplasia (Dudley *et al.* 1995). There is conflicting evidence to suggest that BMP7 is an inductive factor for nephrogenesis (Vukicevic *et al.* 1996; Dudley *et al.* 1999), although it is suggested by both studies that BMP7 acts as a survival factor for the mesenchyme without causing it to differentiate or apoptose but to stay in a stem cell like state (Godin *et al.* 1999). *Ex vivo* culture

studies have shed some light on the specific roles of BMPs in branching morphogenesis. Exogenous BMP7 added to metanephric organ culture inhibits branching at high doses but promotes branching at lower doses (Piscione *et al.* 1997). BMP2 may also have a role in branching morphogenesis. It is expressed from the early aggregate stage of the developing nephrons and inhibits branching and growth of the ureteric bud in culture (Piscione *et al.* 1997). *Bmp2* null mice die prior to metanephric development so it is unclear if a kidney phenotype would manifest (if the kidney were the only affected tissue). Similar affects are seen in cell culture models of branching morphogenesis with BMP7 promoting and BMP2 inhibiting branching in mIMCD$_3$s (Piscione *et al.* 1997; Gupta *et al.* 1999).

Bmp4 also plays a role in controlling branching morphogenesis in the metanephric kidney. Its expression is restricted to stroma surrounding the distal ureter and the Wolffian duct (Miyazaki *et al.* 2000) and is downstream of sonic hedgehog which is expressed by the adjacent epithelium of the distal collecting duct (Yu *et al.* 2002). It specifically inhibits ectopic branch formation while promoting growth and elongation of the stalks. BMP4 negatively regulates GDNF signalling which ensures branch formation only occurs at appropriate sites (*i.e.* the periphery of the kidney) (Miyazaki *et al.* 2000). *Bmp4* null mice die before E10 (Winnier *et al.* 1995). Heterzygotes do survive longer but present with a range of renal defects including hypo/dysplastic kidneys, hydroureter, ectopic ureterovesical junction, and double collecting system (Miyazaki *et al.* 2000).

1.6.1.6 Retinoic acid

Deficiency in vitamin A or retinoic acid during gestation can lead to a cohort of malformations including urogenital defects (Mendelsohn *et al.* 1994). Even a mild reduction in maternal dietary vitamin A can lead to a decrease in nephron endowment (Mendelsohn *et al.* 1994; Vilar *et al.* 1996; Lelievre-Pegorier *et al.* 1998; Mendelsohn *et al.* 1999; Gilbert 2002). In agreement with these findings exogenous Vitamin A in kidney organ culture increases branching morphogenesis (Vilar *et al.* 1996). Compound knockouts for the retinoic acid nuclear receptors (*Rar*) also present with a range of kidney defects (Mendelsohn *et al.* 1994). The stromal

expressed receptors *Rarα* and *Rarβ2* are particularly important for collecting duct development as they help to maintain *Ret* expression at the tips of the branching ureter without which morphogenesis would not proceed properly (Vilar *et al.* 1996; Mendelsohn *et al.* 1999; Gilbert 2002).

1.6.1.7 Fibroblastic growth factors (FGF)

Many FGFs are expressed by the developing kidney although only a few have been implicated in regulating branching morphogenesis (Cancilla *et al.* 1999). These factors signal through receptor tyrosine kinases. FGF7 (keratinocyte growth factor) has an interesting affect on the growth of isolated ureteric buds, inducing the formation of numerous amorphous buds which show little distinction between stalks and tips (Qiao *et al.* 2001). In contrast FGF1 induces the formation of long stalks with distinctive tips which suggests that different FGFs can mediate branching morphogenesis in different ways (Qiao *et al.* 2001). Exogenous FGF7 in organ culture enhances branching of the kidney and knockout mice also present with mild hypoplasia with decreased nephron number (Qiao *et al.* 1999b). The mild nature of this phenotype could be due to functional redundancy between FGFs as more severe defects are seen when signalling through the receptor FGFR2 IIIb, though which 4 FGFs including FGF7 signal, is inhibited in a dominant negative fashion (Celli *et al.* 1998; Cancilla *et al.* 1999).

FGF2 (basic fibroblastic growth factor) has been shown to be involved in the early inductive events of nephron formation (Perantoni *et al.* 1995). It is known to act similarly but to less effect as FGF7 on isolated ureteric bud culture, with the formation of short amorphous ampullae (Qiao *et al.* 2001). The *Fgf2* knockout has no renal defects (Ortega *et al.* 1998).

Recent studies using knockout and knockdown models for *Fgf8* have highlighted two important roles for this FGF in nephrogenesis. FGF8 enhances the survival of developing tubule cells of the nephron. A decrease in *Fgf8* levels results in the production of nephrons with short tubular segments (Grieshammer *et al.* 2005). It is thought that FGF8 is involved in maintaining the progenitor stem cell population at the periphery of the metanephros (Grieshammer *et al.* 2005).

1.6.1.8 Pleiotrophin and midkine

Pleiotrophin and midkine are cytokines with 50% sequence homology (Muramatsu 1993). Identified from conditioned medium of the BSN metanephric mesenchyme cell line, pleiotrophin localizes to the basement membrane of the ureteric bud and is capable of inducing branching of immortalized ureteric bud cells in 3D matrix culture (Sakurai *et al.* 2001). As of yet no knockout has been produced. Midkine, which is expressed in response to retinoic acid signalling (Nakamura *et al.* 1998; Vilar *et al.* 2002), localizes to the basement membrane of both the ureteric bud and the condensing mesenchyme. *In vitro* culture experiments suggest midkine has a role in nephron formation with little effect on branching morphogenesis (Vilar *et al.* 2002). The knockout however has no renal defects (Nakamura *et al.* 1998).

1.6.2 Transcription factors

1.6.2.1 lim1 (Lhx1)

This transcription factor is a member of the LIM class homeobox gene family in the mouse (Fujii *et al.* 1994). *lim1* is expressed initially at E7.5 in the intermediate mesoderm and its expression becomes restricted to the developing nephric duct at E9.5 (Fujii *et al.* 1994). During metanephric development it is expressed weakly in the ureteric bud tips but strongly in the pretubular aggregates of the developing nephrons (Fujii *et al.* 1994). Expression persists in the comma and s-shaped bodies and Bowman's capsule. Most *lim1* null mice die before kidney development at about E10 but the few that survive lack a ureter and metanephros. Using conditional knockout studies it seems that lim1 has multiple roles in renal morphogenesis (Kobayashi *et al.* 2005). When *lim1* is specifically knocked out in the ureteric bud renal hypoplasia results due to severely disrupted ureteric bud branching. Mutants do not show up regulation of *Ret* in the tips of the bud although *Wnt11* expression is maintained (Kobayashi *et al.* 2005).

When *lim1* expression is knocked out in the mesenchymal compartment of the metanephros, hypoplasia results and the underlying defect lies not with ureteric bud

branching but in the inability of the nephrons to progress past the renal vesicle stage. They simply degenerate (Kobayashi *et al.* 2005). It is also suggested that LIM1 may act downstream of WNT4 in early nephron formation (Kobayashi *et al.* 2005).

1.6.2.2 Eya1

Eya1, a transcriptional coactivator homologous to the *Drosophila eyes absent* gene, is critical for the formation of a competent metanephric blastema. Knockout studies in mice identified defects that reflect the human Branchio-Oto-Renal syndrome including renal agenesis (Xu *et al.* 1999). Expression at E9 is found in the caudal region of the intermediate mesoderm just lateral to the Wolffian duct (which expresses *lim1*) and at E11.5 it remains expressed in the metanephric mesenchyme surrounding the ureteric bud (Xu *et al.* 1999; Sajithlal *et al.* 2005). In the knockout for *Eya1*, *Gdnf* expression is lost and *Pax2* expression is reduced specifically in the metanephric mesenchyme (Sajithlal *et al.* 2005). As a result the ureteric bud fails to invade the metanephric mesenchyme but the Wolffian duct seems to be fully competent to branch in response to exogenous GDNF (Sajithlal *et al.* 2005). EYA1 is therefore thought to have an important role in specifying the metanephric blastema within the intermediate mesoderm field.

1.6.2.3 Six genes

Six genes are transcription factors and are homologues of the *Drosophila sine oculis* gene. Two of the six mammalian *Six* genes, *Six*1 and *Six*2, have been investigated in relation to kidney development (Xu *et al.* 2003). Both genes are expressed in the uninduced and induced metanephric mesenchyme and *Six1* expression also comes on later in development in a subset of collecting ducts (Xu *et al.* 2003; Brodbeck *et al.* 2004). A knockout for *Six2* has yet to be created but it has been shown that SIX2 activates transcription of itself and in conjunction with the transcriptional activator EYA1 also activates *Gdnf* expression (Brodbeck *et al.* 2004).

Six1 knockouts lack kidneys due to the failure of invasion of the ureteric bud, which causes default apoptosis of the metanephric mesenchyme (Xu *et al.* 2003). The knockout mesenchyme is unable to undergo nephron formation. *Six1*$^{-/-}$ mice lack *Pax2*, *Six2*, *Sall1* expression in the metanephric mesenchyme although *Eya1* expression is normal (Xu *et al.* 2003). A reduced domain of expression is seen for *Wt1*, *Bmp7* and *Gdnf* (Xu *et al.* 2003). The reduced level of *Gdnf* is suggested to cause the incomplete invasion of the ureteric bud (Xu *et al.* 2003). SIX1 also acts synergistically with EYA1 as compound heterozygotes of both of these genes present with more severe defects than single heterozygotes (Xu *et al.* 2003).

1.6.2.4 Pax2

PAX2 is a transcription factor expressed in the metanephric mesenchyme and also the ureteric bud. There has been conflicting information on the role of PAX2 in regulating *Gdnf* expression in the mesenchyme. Firstly, in *Pax2* knockouts there is no *Gdnf* expression and evidence suggests that *Pax2* itself can activate *Gdnf* expression (Brophy *et al.* 2001). However, in *Wt1* knockouts (Kreidberg *et al.* 1993; Donovan *et al.* 1999) and *Six1* knockouts (Xu *et al.* 2003) *Pax2* expression is lost but *Gdnf* expression is detected albeit in a reduced area. Possible reasons for such results could be that *Pax2* is needed for the initial upregulation in *Gdnf* but its maintanence is independent of *Pax2* or that *Pax2* expression is independent of induction from the ureteric bud (Xu *et al.* 2003).

1.6.3 Extracellular matrix molecules

A number of extracellular matrix molecules have been found to regulate kidney development (reviewed in Wallner *et al.* 1998). Laminin 5 and the laminin binding integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ are important for branching morphogenesis (Zent *et al.* 2001; Chen *et al.* 2004). However collagen binding integrins do not seem to be expressed during early kidney development and this possibly explains the inability of the ureteric bud to branch in a matrix consisting solely of collagen type I (Chen *et al.* 2004). The extracellular matrix molecule fibronectin induces tubule

formation in ureteric bud cells embedded in collagen type 1, again through integrin signalling (Ye *et al.* 2004). *Collagen XVIII* is expressed by the stalk of the ureteric bud (Lin *et al.* 2001). The protein is cleaved to form endostatin, which negatively regulates isolated ureteric bud branching and is thought to signal through glypican receptors (Karihaloo *et al.* 2001).

Sulphated proteoglycans, particularly those containing heparan sulfate glycosaminoglycans (Steer *et al.* 2002), are also crucial to kidney morphogenesis. Inhibition of sulphated proteoglycan synthesis in organ culture affects branching while nephrogenesis is unaffected (Davies *et al.* 1995) (refer to chapter 3).

1.7 Signalling pathways

The numerous factors implicated in regulating branching ultimately have to control intracellular activities of the ureteric bud. Studies on cell culture and organ culture models of kidney development suggest that activation of RET in the ureteric bud epithelium signals through the phosphatidylinositol 3-kinase pathway (Tang *et al.* 2002). The Erk MAP kinase signalling pathway is also involved in modulating GDNF signalling and when this pathway is blocked, the ureteric bud is inhibited from undergoing branching morphogenesis (Fisher *et al.* 2001). Another intracellular signalling pathway, Protein kinase A, modulates signalling by BMPs and activation of this pathway can inhibit branching morphogenesis (Gupta *et al.* 1999). Cytoskeletal proteins such as actin and myosin are involved in cellular morphogenesis and the disruption of either actin or myosin activity results in the inhibition of ureteric bud branching (Michael *et al.* 2005).

1.8 Intrinsic mechanisms of ureteric bud branching

With the development of techniques to culture ureteric bud independently of mesenchyme many authors believe there is an intrinsic mechanism of branching morphogenesis programmed within the ureteric epithelium itself (Dressler 2002; Dziarmaga *et al.* 2003; Meyer *et al.* 2004). It is possible that the cells of the ureteric bud self-organise and regulate their own branching behaviour. It seems in fact that

the ureteric bud can branch in artificial matrices in a manner which is quite reflective of the pattern of branching formed in mesenchyme (Meyer *et al.* 2004).
Studies are beginning to focus on the basic morphogenetic changes taking place within the cells of the ureteric bud (Meyer *et al.* 2004; Michael *et al.* 2004; Michael *et al.* 2005). Although it is clear that branching processes must occur in a spatially and temporally organised manner so that a normal adult renal architecture is formed, it is still unclear how branching activity of the cells of the ureteric bud is controlled.

The ureteric bud is composed of at least two populations of cells (described further in the introduction section of chapter 3), the tip cells and the stalk cells, which exhibit distinct behaviour and cell identity. How the cell identity of these cells is regulated and how they are induced to change their behaviour (from actively branching to a state where branching is inhibited) is unclear. Little is known of why the stalk cells of the ureteric bud do not undergo branching morphogenesis. Also unclear is whether the intrinsic branching program of the ureteric bud includes control programs to ensure that tips of the ureteric bud to not bump into each other as they space out in the mesenchyme. I investigate these aspects of kidney development in order to understand more clearly the intrinsic branching program of the ureteric bud.

I have used micromanipulation and dissection of organ cultured mouse kidneys to investigate the cells of the ureteric bud in relation to their identity and branching behaviour. In this thesis;

- I introduce *Dolichos biflorus* agglutinin as novel marker of the stalk regions of the ureteric bud
- Using this marker will I investigate how cell identity of the ureteric bud is regulated when branching morphogenesis is or is not occurring.
- I investigate the intrinsic abilities of the ureteric bud cells to regulate their behaviour and cell identity. I question whether branches rarely arise from the stalks of the ureteric bud because they have lost this intrinsic ability to branch and how may this ability to branch may be regulated.

- Complementary to this I examine how the branches of the ureteric bud regulate their spacing and positioning. I examine whether the tips of branches repel the presence of neighbouring tips.

Chapter 2

Materials and Methods

2.1 Organ culture

2.1.1 Solutions for organ culture

Culture medium consisted of 100ml of Eagle's minimum essential medium (MEM; Sigma) to which 10ml of fetal calf serum and 1ml of penicillin-streptomycin stabilized solution (100x) (Sigma) and was stored at 4°C. 30mM $NaClO_3$ in culture medium was made up fresh for use. Trypsin medium was prepared with MEM mixed with Trypsin-EDTA solution (Sigma) in a ratio of 4:1. The lectin from *Dolichos biflorus* was obtained from Sigma. It was stored at -20°C and was made as 1mg/ml in MEM. This stock was added to some organ cultures to obtain a final concentration of 10μg/ml.

2.1.2 Kidney dissections and culture

Metanephric kidneys were dissected from E11.5/E12.5 embryos from either MF1 or CD1 outbred mice (this change in mouse strain was due to relocation of the animal house facilities). MF1 mice were used for most of the DBA time course cultures while regenerating stalk cultures, clustered kidney experiments and $NaClO_3$ experiments were from CD1 mice. The morning when the vaginal plug was discovered was deemed to be E0.5. Dissections were carried out in 35mm petri dishes in minimum essential medium Eagle (MEM; Sigma) using 0.5x16m needles (BD Microlance 3). Kidneys or microdissected rudiments were cultured for a period of time depending on the specific experiment. Kidneys were placed on top of either 5μm or 0.1μm isopore membrane filters (Millipore) on metal grids in petri dishes (figure 2.1). The dish was filled with culture medium (100ml of MEM with 10ml of fetal calf serum and 1ml of penicillin-streptomycin solution stabilized (100x) (Sigma)) just below the level of the grid. All cultures were incubated in a humidified incubator with 5% CO_2 at 37°C.

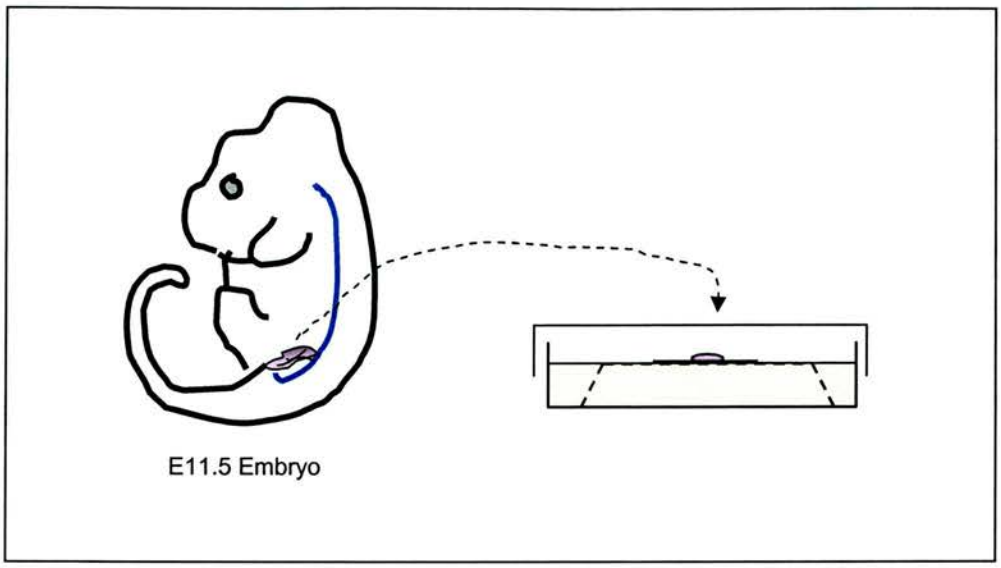


Figure 2.1: Organ culture method

2.1.3 $NaClO_3$ culture

Kidneys were dissected from E11.5 embryos as described earlier. This experimental plan was set up twice each time. One set of kidneys was processed for *Wnt11 in situ* hybridisation and the other set was used for lectin histochemistry/immunohistochemistry using anti-calbindin-D_{28k} and *Dolichos biflorus* agglutinin (DBA). Filters were cut into specific shapes to identify the various samples.

Isopore membrane filters of pore size 0.1μm were used for all samples. Those samples cultured in 30mM $NaClO_3$ medium were cultured in the same dish for 24hr (table 2.1). The samples receiving a recovery period were transferred to a dish of MEM to wash away any chlorate residue and were floated off their filter. They were assigned randomly to fresh filters in a new dish of culture medium and a new grid. Culture medium was changed daily.

Table 2.1: Outline of Cultures of E11.5 kidneys with/without 30mM $NaClO_3$

Length of culture in culture medium (hrs)	Length of culture in $NaClO_3$ medium	Length of recovery in culture medium
24hr	-	-
-	24	-
-	24	24
-	24	48
-	24	72

2.1.4 Set up of stalk regenerating culture

E11.5 or E12.5 kidneys were kept in MEM and were microdissected further using needles and a dissecting microscope on high power (x50 magnification). Each kidney was trimmed so that surrounding tissues were fully removed. Any remnants of the Wolffian duct were trimmed away. The stalk was dissected away from the mesenchyme by cutting below where the ureteric bud had undergone its first round of branching morphogenesis. If any mesenchyme remained attached to the stalk itself no attempted was made to remove it. This made the dissected stalk more manageable. The stalk was stored in MEM while the preparation continued.

The remaining part of the kidney now consisted of two tip regions of the ureteric bud, any remaining stalk regions and the surrounding metanephric mesenchyme. These were cultured for three hours before being fixed for either *Wnt11 in situ* hybridisation, which identifies the tip regions of the ureteric bud, or for DBA lectin histochemistry/immunohistochemistry, which identifies the stalk regions of the ureteric bud. The purpose of staining these kidney parts, from which stalk regions were removed, was to demonstrate that the tip regions were left intact and were not included with the removed stalk regions.

Further kidneys were dissected and trimmed by removing most of the stalk region and any Wolffian duct remnant. The mesenchyme was loosened from the embedded

ureteric bud by incubating the rudiments in trypsin medium (1ml of trypsin-EDTA solution (Sigma) with 4ml of MEM) for 30-40 minutes at 37°C in a humidified incubator with 5% CO_2. The rudiment was then transferred to culture medium and the mesenchyme was separated from the ureteric bud tissue within. Stickiness of the rudiment was reduced by adding a small amount of DNase (Promega) to the culture medium (5U of DNase per ml of culture medium). The mesenchyme was collected by pipetting and stored in MEM. The ureteric buds were discarded.

The mesenchyme of approximately 10 kidneys was transferred by pipetting to 0.1μm isopore filters on metal grids in petri dishes with culture medium. The mesenchyme was allowed to settle on the filters by leaving it for 40-60 minutes at 37°C in a humidified incubator with 5% CO_2. The mesenchyme was nudged into a ball using needles. A single stalk rudiment was then transferred to the filter and after settling it was made, using needles, to closely appose and sit into the mesenchyme ball. Cultures were left for 144hr. Culture medium was changed daily.

2.2 Cell Culture

mIMCD$_3$ cells (ATCC number: CRL-2123) were cultured in a 1:1 Ham's F12 (Sigma) and Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% Penicillin/Streptomycin (Sigma). Cells were fixed and stained according to the methods outlined for cultured kidneys.

2.3 Lectin histochemistry/immunohistochemistry

2.3.1 Fixation of kidneys for lectin histochemistry/immunohistochemistry

Kidneys processed for lectin histochemistry/immunohistochemistry were fixed as follows. In order to fix them to their filters the culture medium was aspirated off and replaced with 100% ice cold methanol. This was left for up to 5 minutes at room temperature. The kidneys, now attached to their filters, were transferred to labelled bijoux with 100% ice cold methanol for long term storage at –20°C.

2.3.2 Lectin histochemistry/immunohistochemistry for Laminin and DBA

Filters with kidneys were washed for the 30 minutes in 1% milk powder (SMA) in PBS at room temperature while agitating gently. They were stained in rabbit anti-laminin (Sigma) diluted 1:100 in 1% milk powder in PBS overnight at 4°C. Another wash was carried out for 30 minutes in 1% milk powder in PBS at room temperature while agitating gently. They were then transferred to goat anti-rabbit IgG (whole molecule)-TRITC (Sigma) diluted 1:100 and lectin from *Dolichos biflorus* (horse gram)-FITC (Sigma) diluted 10ng/ml (1:100 of 1mg/ml PBS stock) in 1% milk powder in PBS overnight at 4°C. A final wash for five minutes was carried out again in 1% milk powder in PBS at room temperature while agitating gently. The filters were then mounted onto glass slide using the aqueous mountant mowiol and were imaged by confocal microscopy.

2.3.3 Lectin histochemistry/immunohistochemistry for calbindin-D_{28k} and DBA

Filters with kidneys were washed for the 5 minutes in 4% milk powder (SMA) in PBS at room temperature while agitating gently. They were incubated in mouse anti-calbindin-D_{28k} (Abcam) diluted 1:100 in 4% milk powder in PBS overnight at 4°C. Another wash for 5 minutes was carried out in 4% milk powder in PBS at room temperature while agitating gently. They were then transferred to donkey anti-mouse IgG-Texas Red (Abcam) diluted 1:100 and lectin from *Dolichos biflorus* (horse gram)-FITC (Sigma) diluted 10ng/ml (1:100 of 1mg/ml PBS stock) in 4% milk powder in PBS overnight at 4°C. A final wash for 30 minutes was carried out again in 4% milk powder in PBS at room temperature while agitating gently. The filters were then mounted onto glass slides using the aqueous mountant mowiol and were imaged by confocal microscopy. A summary of the stains and antibodies used is presented in table 2.2.

Table 2.2: Summary of Antibodies and Lectins used for staining

Antibody Name	Manufacturer	Catalogue number	Dilution in 4% milk powder in PBS
Rabbit anti-laminin	Sigma	L9393	1:100
Goat anti-rabbit IgG (whole molecule)-	Sigma	T5268	1:100
Mouse anti-calbindin-$_{D28k}$	Abcam	ab9481	1:100; for the staining in chapter 3 a dilution of 1.5:100 was used.
Donkey anti-mouse IgG-Texas Red	Abcam	ab7059/ ab6818	1:100
Nuclear stain	**Manufacturer**	**Catalogue number**	**Dilution used**
TO-PRO-3	Invitrogen Molecular probes	T3605	1:250
Lectin Name	**Manufacturer**	**Catalogue number**	**Dilution used**
Lectin from *Dolichos biflorus* (horse	Sigma	L9142	10ng/ml (1:100 of 1mg/ml PBS stock)

2.4 Microbiology

2.4.1 Solutions for microbiology

Ampicillin stock solution consisted of 50mg of ampicillin sodium salt dissolved in 1ml of ethanol, which was aliquoted and stored at -20°C.

Luria-Bertani medium (LB) with/without ampicillin was composed of tryptone (Sigma) 10g, Yeast Extract (Sigma) 5g, NaCl (Sigma) 5g, dissolved in distilled water to 1L. The pH of this solution was adjusted to 7.5 with NaOH and it was microwaved to sterilize it. At this stage all solvents were fully dissolved and the solution was

straw yellow in colour. The solution was placed in an aseptic environment to cool. When needed, ampicillin solution was added to give a final concentration of 100ug/ml. The solution was cooled to no more than 55°C before ampicillin was added. Solution was made up fresh before use.

To prepare LB agar, 15g of agar was added to fresh LB medium solution just prior to microwaving. The agar solution was poured into Petri dishes under aseptic conditions and these were set with their lids on at 4°C until needed for plating of *E. coli.* Fresh solution was made up for use each time.

For use with *E. coli* transformations SOC medium was prepared with 2g of Tryptone (Sigma), 0.5g Yeast extract (Sigma), 1ml 1M NaCl (Sigma), 0.25ml 1M KCL (Sigma), added to 97ml of distilled water. These were stirred until fully dissolved before being microwaved to sterilise them. After the solution cooled to room temperature, 1ml of Mg^{2+} stock (10.15g $MgCl_2 \cdot 6H_2O$, 12.33g $MgSO_4 \cdot 7H_2O$, up to 50ml with distilled H_20; this solution was passed through a 0.2μm filter unit and stored at room temperature) and 1ml of 2M glucose solution (18.02g glucose, up to 50ml with distilled H_20, this solution was passed through a 0.2μm filter unit, stored at -20°C and was assumed stable for 1 year) were added. The final solution was filtered through a 0.2μm filter unit and pH was adjusted to 7.0. Fresh solution was made up for use.

2.4.2 *Wnt11* Plasmid

The *Wnt11* plasmid used to generate *Wnt11* probes for *in situ* hybridisation has been used elsewhere (Kispert *et al.* 1996) and was kindly donated by S. Vainio (University of Oulu, Finland). It consisted of a 2.1kb cDNA of *Wnt11* from a newborn mouse kidney library which was cloned into a pSK II plasmid. This plasmid carried the ampicillin resistance gene.

2.4.3 Transformations

The *Wnt11* plasmid was transfected into competent *E. coli* JM109 cells (Promega) by heat shock. Sterile polypropylene culture tubes were chilled on ice. The bacterial cells were removed from storage from the -70°C and were kept on ice until thawed. The cells were gently mixed by flicking the tube and 100μl of the *E. coli* solution was transferred to a chilled culture tube. 10μl of *Wnt11* plasmid solution (eluted from spot of plasmid on filter paper into 1ml) was added to the *E. coli* solution. The tube was flicked quickly several times to mix the contents. It was immediately returned to ice for 10min and then submerged in a water bath at 42°C for 70 seconds. The culture tube was handled carefully to avoid shaking and it was immediately returned to ice for 2min. 900μl of cold (4°C) SOC medium was added to the tube and this was incubated at 37°C for 2hr while agitating at 225rpm in an orbital shaker. 20μl of this transformation solution was then plated onto a LB agar plate (with ampicillin included) and spread. After plating the culture was left upside down overnight at 37°C. Transformations using the control plasmid provided by Promega were carried out as well as an experimental control.

2.4.4 Isolation of plasmid DNA

The following day a few colonies were selected and were used to inoculate 2 x 25ml LB with ampicillin solution in 50ml falcon tubes. The solutions were left overnight at 37°C in an orbital shaker. The following morning the optical density (λ = 600) of the *E. coli* solution was checked with a spectrophotometer so ensure the *E. coli* solution was somewhere in the lag phase (approximately 0.6 OD). One of the 25ml cultured solutions was pelleted by spinning in a centrifuge and the pellet was processed further using a Miniprep kit (Qiagen) according to manufacturer's instructions. The second 25ml culture solution of each colony was used to create glycerol stocks. A sample of the isolated DNA was analysed by gel electrophoresis to check size and estimate its concentration.

2.4.5 Production of Bacterial stocks

When the *Wnt11* plasmid was successfully transformed into *E. coli* by heat shock glycerol stocks of the transformed *E. coli* were created. A 25ml culture of the successfully transformed colony was spun by centrifugation and the *E. coli* pellet was harvested and resuspended in 1ml of LB. 800µl of this solution was added to 200µl of 80% glycerol (80ml of glycerol added to 20ml of H_2O, autoclaved and stored at room temperature) and it was stored at -70°C in 1.5ml cryotubes.

2.5 Gel Electrophoresis

DNA Gel electrophoresis was performed to check the size and estimate the concentration of both DNA and RNA. A 1.5% agarose gel (Invitrogen) was prepared made using 1 x TBE (Sigma). The solution was microwaved until all the agarose was fully dissolved. Ethidium bromide solution was added to obtain a final concentration of approximately 500ng/ml of agarose. The gel solution was poured into a sealed gel tray and allowed to set either at room temperature or at 4°C. Samples were always run with a sample of a 100bp DNA ladder (Promega) as a size reference. DNA samples were loaded using 6x blue/orange loading dye (Promega).

RNA gel electrophoresis was performed similarly; except DEPC treated TBE was used to make the gels. The gel tank was decontaminated before use by rinsing in 0.1% SDS solution overnight and washing in dH_20 before use. RNA samples were loaded using RNA loading dye (Ambion) and the 100bp DNA ladder (Promega) was run along side as a reference. Gels were visualised using a ultraviolet trans-illuminator and were photographed.

2.6 Riboprobe synthesis

2.6.1 DNA plasmids for generation of riboprobes for *in situ* hybridisation

The *Wnt11* plasmid as outlined above was used to generate sense and antisense riboprobes following appropriate restriction enzyme digestions which can be summarised as in table 2.3.

Plasmid	Antisense riboprobe		Sense riboprobe		Probe length
	Restriction enzyme	Polymerase Enzyme	Restriction enzyme	Polymerase Enzyme	
Wnt11	XhoI	T_3 polymerase	XbaI	T_7 polymerase	1.7kb

Table 2.3: Summary of the reactions involved in synthesising *Wnt11* riboprobes

2.6.2 Restriction enzyme digest

Plasmid DNA was linearized using appropriate restriction enzymes from either Sigma or Promega. Digests were carried out as to the manufacturers recommended protocol. The reactions were incubated for 2hr at 37°C using a heating block. Following the digestion of the linearized DNA it was precipitated by adding 10μl of 3M $C_2H_3NaO_2$ followed by 250µl of ice cold 100% ethanol. This was left at -20°C overnight. The following morning the solution was centrifuged for 15min at 13,000 rpm. The pellet was resuspended in 50μl of 70% ethanol and centrifuged again at 13,000rpm for 5min. The ethanol was removed and the pellet was left to air-dry for a few minutes. It was suspended in 20μl of dH_20. The concentration of the solution was estimated by gel electrophoresis of 1μl.

2.6.3 Synthesis of DIG labelled riboprobes

Digoxigenin (DIG) labelled riboprobes were synthesised for *in situ* hybridisation. Degradation by RNases was avoided by keeping all solutions on ice

and by wearing gloves at all times. Synthesis reactions were set up to a final volume of 20μl (8μl dH_2O, 2μl transcription buffer, 5μl linearized *Wnt11* plasmid DNA (~200ng/μl), 2μl NTP labelling mixture (with DIG-UTP), 1μl RNasin RNase inhibitor (Promega) and 1μl of either T_3 or T_7 DNA polymerase (20U/μl)). The reaction was mixed by pipetting and left for 2 hours at 37°C. 2μl of RNase-free DNase1 (Promega) was added for 15 minutes incubation again at 37°C. To stop the reaction, 0.2M EDTA, pH8.0 was added. The RNA was precipitated by adding 2.5μl of 4M LiCl followed by 75μl of 100% ethanol. This was left overnight at -20°C. The following day the solution was spun in a microcentrifuge at 13,000rpm for 10 minutes. The supernatant was carefully removed and discarded. The pellet was resuspended in 70% ice cold ethanol and was spun again at 13,000rpm for 10 minutes. After the supernatant was removed the pellet was allowed to air dry for a few minutes at room temperature before resuspension in 110μl dH_20 with 1μl RNasin RNase inhibitor. RNase free gel electrophoresis was carried using 1μl of the RNA solution to check the integrity of the RNA and to estimate its concentration. The solution was diluted in prehybridisation solution to which Protect RNA (Sigma) had been added. It was aliquoted and stored at -70°C.

2.7 RNA *in situ* hybridisation

2.7.1 Solutions for RNA *in situ* hybridisation

DEPC treated water/PBS consisted of 1ml of diethyl pyrocarbonate (DEPC), added to 1L of either water or PBS (Sigma). The solution was left to stand overnight at 37°C before autoclaving and storing at room temperature. dPBT was made from DEPC treated PBS to which 0.1% Tween20 (Sigma) was added. This was stored at room temperature.

To prepare 4% PFA, 4g of paraformaldehyde were added to 100ml of DEPC- treated PBS. The solution was heated to 65°C until fully dissolved and it was aliquoted and stored for up to three months at -20°C.

Torula yeast tRNA solution consisted of approximately 45ml of DEPC-treated water which was microwaved until boiling, after which 3g of torula yeast tRNA powder

(sigma) was added. It was dissolved by shaking, allowed to cool and centrifuged. Any undissolved yeast tRNA was discarded. The concentration of RNA in the solution was measured using a spectrophotometer and the concentration was adjusted accordingly to achieve a solution of 50mg/ml. The solution was aliquoted and stored at -20°C until used. 20x SSC solution was prepared using 175.3g of sodium shloride and 88.2g sodium citrate in 800ml of DEPC treated water. pH was adjusted to 5 with concentrated NaOH. The volume was adjusted to 1L and the solution was stored at room temperature 0.2x SSC was prepared as a 1:10 dilution of 20x SSC and 0.2X SSC was similarly prepared as a 1:10 dilution of 2x SSC.

2.7.2 Fixation of kidneys for *in situ* hybridisation

Kidneys processed for *in situ* hybridisation were fixed as follows. In order to fix them to their filters the culture medium was aspirated off and replaced with 100% ice cold methanol. This was left for 5 minutes so that kidneys would fix to their filters. The filters (with attached kidneys) were transferred to labelled bijoux with 4% PFA and were kept overnight at 4°C before being transferred to 100% ice cold methanol for long term storage at –20°C.

2.7.3 RNA *in situ* hybridisation protocol

Kidneys on the filters were processed for *in situ* hybridisation based on Wilkinson's protocol (Wilkinson 1992). They were briefly rehydrated through a series of methanol in dPBT (Diethyl Pyrocarbonate treated PBS, 0.1% Tween20 (Sigma)) for 10 minutes on ice. After washing in dPBT 3 x 5 minutes on ice, kidneys were digested with 10µg/ml proteinase K (Sigma) in dPBT for 15 minutes at room temperature. Kidneys were washed 3 x 5 minutes in dPBT before being fixed for 40 minutes in 4% PFA on ice. Rinsing 3 x 5 minutes in dPBT preceded prehybridisation. Prehybridisation solution consisted of 50% deionised formamide (Ambion), 25% 20x SSC, 2% blocking powder (Roche), 0.1% Tween20, 0.5% CHAPS (Sigma), 1mg/ml Yeast RNA (Sigma), 0.5M EDTA, 0.05mg/ml heparin (Sigma). Kidneys were prehybridised in this solution for 2-4 hours at 65°C before the

probe was added. The probe at a final concentration of 250ng/ml was heated for three minutes to 80°C before adding it to the kidneys. Incubation with the probe was carried out at 60°C overnight. Protect RNA (Sigma) was used in all solutions after digestion with proteinase K until the end of hybridisation.

The following day kidneys were washed in 100% post-hybridisation solution (50% deionised formamide, 25% 20x SSC, 0.1% Tween20, 0.5% CHAPS) 2 x 10 min at room temp. They were then washed in 75% post-hybridisation solution/2x SSC, 50% post-hybridisation solution/2x SSC, 25% post-hybridisation solution/2x SSC each for 10 min at room temperature.

Washes in 2x SSC, 0.1% CHAPS and 0.2x SSC, 0.1% CHAPS were carried out for 2 x 30 minutes each at 65°C. Kidneys were then equalised with TBST (TBT and 0.1% Tween20) before being blocked for 2-4 hr at 4°C in TBST with 10% sheep serum (Sigma) and 2% bovine serum albumin (Sigma). Samples were left overnight at 4°C in 1:2000 alkaline phosphatase, conjugated anti-DIG antibody (Roche) in fresh blocking solution.

Excess antibody was washed away by rinsing the kidneys 6 x 15 minutes in TBST with 0.1% Bovine serum albumin followed by 2 x 30 minute washes in NMT (100mM NaCl, 100mM Tris-Cl (pH 9.5), 50mM $MgCl_2$) with 0.1% Tween20. Further washes in NMT, 3 x 15 minutes were carried out before colour development in staining solution of 17µl/ml NBT/BCIP solution (Roche) in NMT. After colour development in the dark the kidneys were fixed in 100% methanol before mounting on slides with mowiol (Calbiochem).

2.8 Microscopy

2.9 Solutions for microscopy

Mowiol mounting medium was prepared from 2.4g of Mowiol (Calbiochem) and 4.76ml glycerol (Sigma) which were placed into a 50ml conical flask and stirred with a pipette to mix. 12ml of H_20 was added and the mixture was left overnight at room temperature. The following day 12ml Tris (pH8.5) was added. The solution was heated to 50°C for 2hr with occasional vortexing. The solution was centrifuged at

2000rpm for 15 minutes before 0.72g of 1,4 diazobicyclooctane (antifade; Sigma) was added. The solution was aliquoted and stored at -20°C. This mountant was used for all slides and it was warmed to room temperature before use. It was also centrifuged before use to remove any bubbles.

2.10 Confocal Microscopy

Samples stained by lectin histochemistry/immunohistochemistry were imaged using the TCS NT Leica confocal laser scanning microscope (Leica Microsystems). Samples were imaged on the TRITC and FITC channels individually to avoid cross talk between the two channels. Serial optical sections were obtained for most kidney samples and stalk regenerating samples. Optical sections were set to a approximately 3μm-5μm thickness unless otherwise stated. Sections were scanned 4 times and averaged. Maximum projection images of a series were presented in the thesis unless otherwise stated.

2.11 *In situ* hybridisation imaging

In situ hybridisation samples were imaged using a Zeiss Axioplan 2 microscope with a Nikon Coolpix 995. A stage micrometer (Graticules Ltd.) was imaged with every collection of images so that scale bars could be accurately inserted.

2.12 Image analysis

Scale bars were inserted and image analysis was carried out using Adobe Photoshop 4.0, Microsoft PowerPoint and Scion image. Leica Confocal Software Lite program version 2 was used to view the images obtained with the confocal microscope. Linda Wilson kindly helped with the use of ImarisSurpass for the generation of the 3D images presented in chapter 3.

2.13 Statistical Analysis

Microsoft Office Excel 2003 and Minitab (release 14, MINITAB Inc) software programs were used to carry out statistical analysis. Gráinne Long graciously helped with the use of Minitab and the statistical analysis. One-way ANOVA comparisons were carried out and where appropriate the data was transformed to meet the assumptions of normality and homogeneity of variances. Transformations included log, square root, cubed or inverse and the appropriate transformation for each analysis was stated in the figure legend. Bar charts and histograms were constructed using Microsoft Office Excel 2003. Where shown, error bars represent the standard error of the mean, * $p< 0.05$ and ** $p<0.001$. Binomial probabilities were calculated using Microsoft Office Excel 2003 and using the online z to p calculator which can be found at the URL http://faculty.vassar.edu/lowry/zp.html

Chapter 3

Dolichos biflorus agglutinin is a marker of non-branching regions of the ureteric bud

3.1 Introduction

Over the last few of years, studies on metanephric branching morphogenesis have focused on identifying intrinsic properties of the ureteric bud which regulate how, when and where new branches form (Michael 2003; Meyer *et al.* 2004). The ureteric bud was thought to be composed of a homogeneous population of cells which express a number of identifying markers such as calbindin-D_{28k}, $P_{CD}9$ and cytokeratin7 (Liu *et al.* 1993; Kloth *et al.* 1998; Brophy *et al.* 2001). However recent investigations have suggested that the epithelium is actually a heterogeneous population of at least two cells, the tip cells and the stalk cells. It appears that the epithelium is strictly patterned with regard to these different cell types and this patterning may be important in controlling the spatial and temporal aspects of branching morphogenesis and, as a consequence, of nephrogenesis. Previous morphological differences have been reported between the epithelia of tips and stalks, with the tip cells taking on a round stratified morphology in contrast to simple columnar appearance of the stalk cells (Qiao *et al.* 1995) although the tips of the ureteric bud have also been described as being wedge shaped compared to the stalks (Meyer *et al.* 2004). Even though these differences have been reported it seems that both tip and stalk cells, at least in isolated ureteric bud culture, are polarised apicobasally (Meyer *et al.* 2004). The tips of the ureteric bud branches are also thought to induce the differentiation of the metanephric mesenchyme and are the regions at which most of the branching events are initiated (Davies *et al.* 2002; Watanabe *et al.* 2004; Yu *et al.* 2004). On the other hand, it is believed that stalk regions do not initiate mesenchyme induction and that few branches arise from these regions. In addition to having different functions, the tips and stalk regions of the ureteric bud express quite a number of genes differentially (Davies 2005; Schmidt-Ott *et al.* 2005). Table 3.1 summarizes a few known differentially expressed genes.

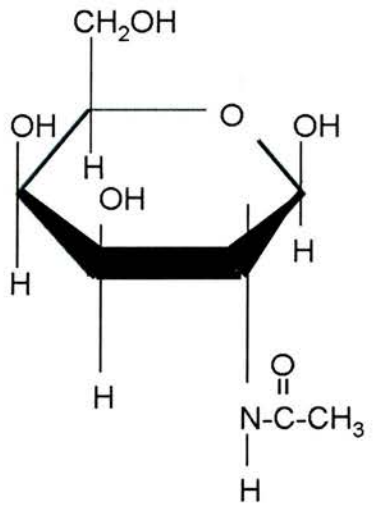
Marker	Localisation/expression in the metanephros	Knockout phenotype	References
Wnt11	Expressed by the tip regions of the ureteric bud	ureteric branching morphogenesis defects and kidney hypoplasia	(Christiansen *et al.* 1995; Majumdar *et al.* 2003)
c-ret	Expressed by the tip regions of the ureteric bud	renal agenesis and dysplasia	(Pachnis *et al.* 1993; Schuchardt *et al.* 1994)
P(CD)Amp1	Localises to the tip region of the embryonic collecting duct	Unknown	(Strehl *et al.* 2001)
Coll XVIII	Expressed by the stalk regions of the ureteric bud	broadened basement membranes of tubules and altered renal filtration capacity	(Vainio *et al.* 2003; Utriainen *et al.* 2004)
P CD 5V	Stalks of ureteric bud	Unknown	(Minuth *et al.* 1989)
P CD 4	Stalks of ureteric bud	Unknown	(Minuth *et al.* 1989)
P CD 1	Stalks of ureteric bud	Unknown	(Gilbert *et al.* 1987)
P CD 2	Stalks of ureteric bud	Unknown	(Gilbert *et al.* 1987)
P CD 3	Stalks of ureteric bud	Unknown	(Gilbert *et al.* 1987)

Table 3.1: Summary of some of the markers of tips and stalks of the ureteric bud.

The markers listed above either localise or are expressed in the tip or stalk of the ureteric bud. It is unclear generally, whether or not these markers all respect the same tip/stalk boundary.

Lectin fluorescence has been used for many years as both a research tool and a diagnostic tool in pathology to study and characterise tissues and cells (Bramwell *et al.* 1982; Walker 1989; Brinck *et al.* 1995; Nakagawa *et al.* 1996; Mitchell *et al.* 1999). Previous work in the laboratory has led to the identification of *Dolichos biflorus* agglutinin (DBA), as a novel marker apparently specific for the stalk regions of the ureteric bud (unpublished work from Michael 2003). DBA is a plant lectin from the *Dolichos biflorus* plant and has been previously used to highlight specific tissue components of organs including the stomach (Petrovic *et al.* 2002), the lung (Barkhordari *et al.* 2004) and kidney collecting duct cells *etc.* (Laitinen *et al.* 1987;

Schumacher *et al.* 2002). Lectins are carbohydrate-binding proteins involved in a variety of recognition processes as they are capable of binding to specific glycosyl residues in a reversible manner (reviewed by Vijayan *et al.* 1999). DBA specifically has affinity for N-acetyl-D-galactosamine residues (Etzler *et al.* 1970; Hammarstrom *et al.* 1977; Hamelryck *et al.* 1999; Schumacher *et al.* 2002) but it is unknown what glycoprotein, or glycoproteins carry these residues in the ureteric bud. The structure of N-acetyl-D-galactosamine is shown below (figure 3.1) (taken from Stryer 1995).



β-D-N-Acetylgalactosamine (GalNAc)

Figure 3.1: The structure of N-acetyl-D-galactosamine.

DBA has been used in previous studies as a marker of the ureteric bud (Barasch *et al.* 1996; Qiao *et al.* 1999a; Legallicier *et al.* 2001) but here I show that DBA has a more restricted binding pattern as suggested in preliminary and unpublished results from Dr. Lydia Michael (of this laboratory). My results suggest that DBA is a useful marker of stalk regions of the ureteric bud.

Having established that DBA is a marker of stalk regions of the ureteric bud, I investigate the identity of cells of the ureteric bud when branching morphogenesis is blocked. Branching morphogenesis in culture can be blocked by interfering with signalling of important molecules such as Glial derived neurotrophic factor (GDNF).

Gdnf has been found to be critical for ureteric bud branching branching (Sanchez *et al.* 1996). GDNF is secreted by the metanephric mesenchyme and signals through the RET receptor and GFRα1 co-receptor (Sariola *et al.* 2003; Sainio *et al.* 1997). The effects of GDNF can be down regulated effectively in kidney organ culture by the addition of GDNF function blocking antibodies (Vega *et al.* 1996; Davies *et al.* 1999; Fisher *et al.* 2001). The addition of sodium chlorate to organ culture blocks branching (Davies *et al.* 1995) and diminishes *Gdnf* expression (Kispert *et al.* 1996). The chlorate ion has a shape similar to sulphate and it inhibits the enzyme ATP sulfurylase (figure 3.2). *In vivo* ATP sulfurylase converts sulphate into PAPS, the active sulphate donor used in the sulphation of glycosaminoglycan. Chlorate inhibits ATP sulfurylase activity and consequently there is a decrease in the levels of sulphated glycosaminoglycan (GAG). Sulphated GAGs are essential for ureteric bud growth and branching but are dispensable for nephrogenesis (Davies *et al.* 1995; Davies *et al.* 2001). GAGs containing 2-O-sulphate groups seem to be of particular importance in regulating ureteric branching (Bullock *et al.* 1998; Davies *et al.* 2003). As sulphated glycoproteins are required for GDNF bioactivity (Barnett *et al.* 2002; Rickard *et al.* 2003), GDNF activity is also rapidly down regulated as a consequence of the addition of chlorate.

When GDNF signalling is reduced, the kidney stops undergoing branching morphogenesis and the expression of tip specific markers such as *Wnt11* is lost (Kispert *et al.* 1996; Majumdar *et al.* 2003). As both branch formation and tip marker expression are halted it may be that the tip regions of the ureteric bud change their cell behaviour. It is not known whether the loss of tip markers is accompanied by a gain of stalk markers, or whether the cells enter a third state, neither 'tip' not 'stalk', when branching morphogenesis is blocked. In order to understand how branching behaviour of the ureteric bud is regulated and coordinated, it is crucial to explore the cell behaviour of the tip and stalk regions. Using two markers exclusively specific for either the stalk regions (*Dolichos biflorus* agglutinin) or tip regions (*Wnt11*) of the ureteric bud, I have evaluated the behaviour of cells of the ureteric bud under conditions where branching morphogenesis is blocked.

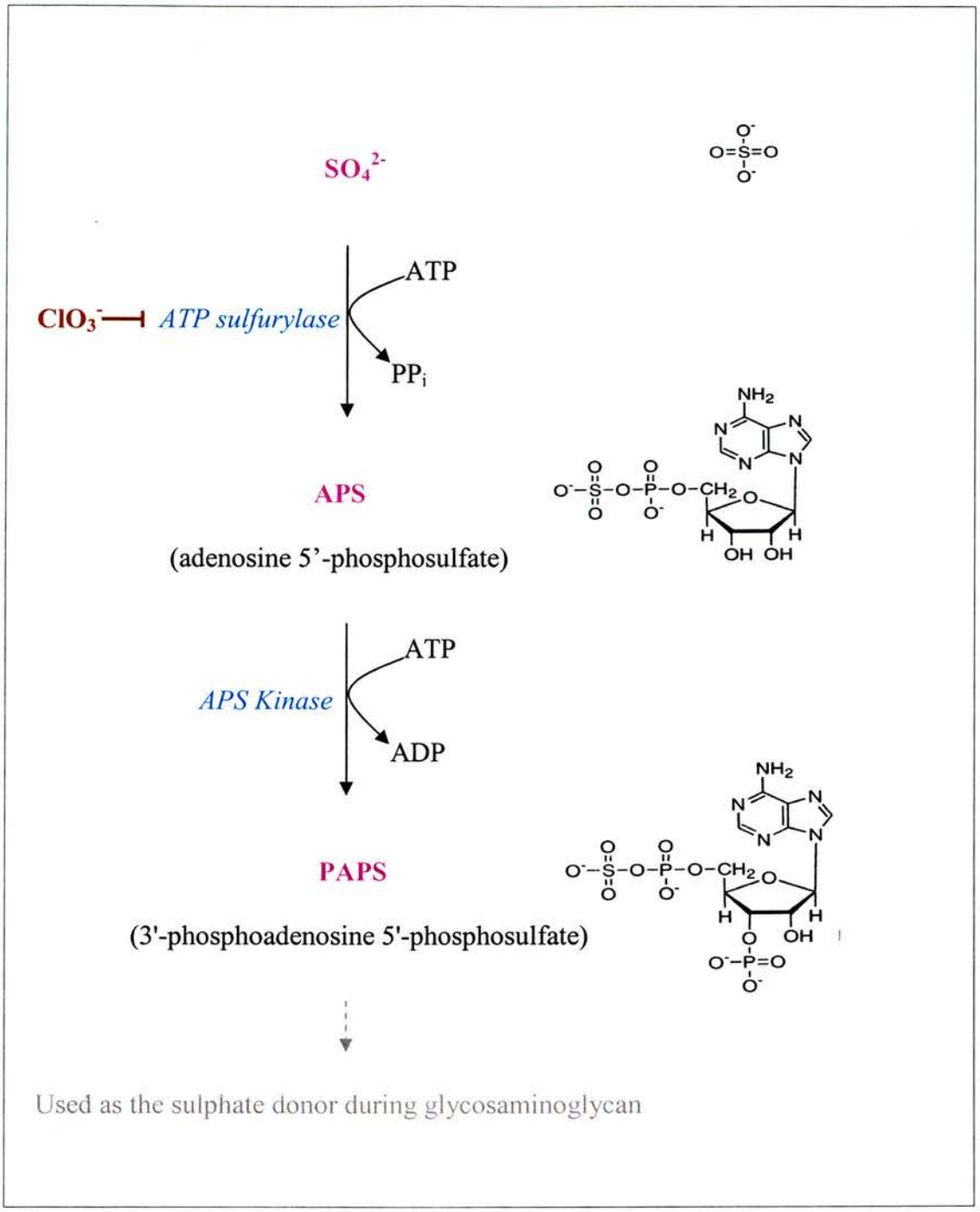


Figure 3.2: Chlorate inhibition of PAPS synthesis.

ATP sulfurylase catalyses the conversion of sulfate into adenosine 5'-phosphosulfate. This process is a key step in the synthetic pathway of 3'-phosphoadenosine 5'-phosphosulfate (PAPS). PAPS is the sulphate donor during glycosaminoglycan sulphation. The chlorate ion inhibits the enzyme ATP sulfurylase and as a consequence the synthesis of sulphated glycosaminoglycans is inhibited. Chemical structures are taken from Leustek *et al.* 1999.

3.2 Results

3.2.1 *Dolichos biflorus* binds to terminally differentiated cells derived from the ureteric bud

The reported binding pattern of DBA in the adult kidney varies somewhat; for example DBA has been reported to bind to cells of the loop of Henle (Engel *et al.* 1997) but in general DBA is usually regarded as binding to collecting duct cells (Laitinen *et al.* 1987). The mouse inner medullary collecting duct cell line ($mIMCD_3$) is derived from the inner medulla of the adult mouse kidney (Rauchman *et al.* 1993). They undergo branching in 3D collagen matrices and are a cell model of branching morphogenesis (Cantley *et al.* 1994). As cells of the adult collecting duct system, $mIMCD_3$ cells are considered to be terminally differentiated cells derived from the ureteric bud. The binding pattern of DBA in $mIMCD_3$ cells was investigated to confirm the affinity of DBA for cells of the adult collecting duct (figure 3.3). $mIMCD_3$ cells were stained with DBA, anti-laminin and the nuclear stain, topro3. DBA bound well to the cells but binding was depressed in regions of the cell nucleoli and was higher in some perinuclear areas. DBA staining was often seen as punctate granules around the nucleus. Anti-laminin was found associated with all cells and the staining was carpet-like although it appeared higher in some perinuclear areas. No co-localization of DBA and anti-laminin was obvious. The binding of DBA was not homogeneous from cell to cell with some cells showing greater binding than others. This heterogeneity of the staining may be accounted for by considering that these images were single section confocal images or that these cells were not clonal.

3D reconstruction of a serial section of images taken of mIMCD3 cells stained with DBA, anti-laminin and topro3 suggested that DBA and anti-laminin bound to the basal or lateral aspects of the cells (figure 3.4). Neither DBA nor anti-laminin bound to the apices of the cells (*i.e.* superior to the nucleus).

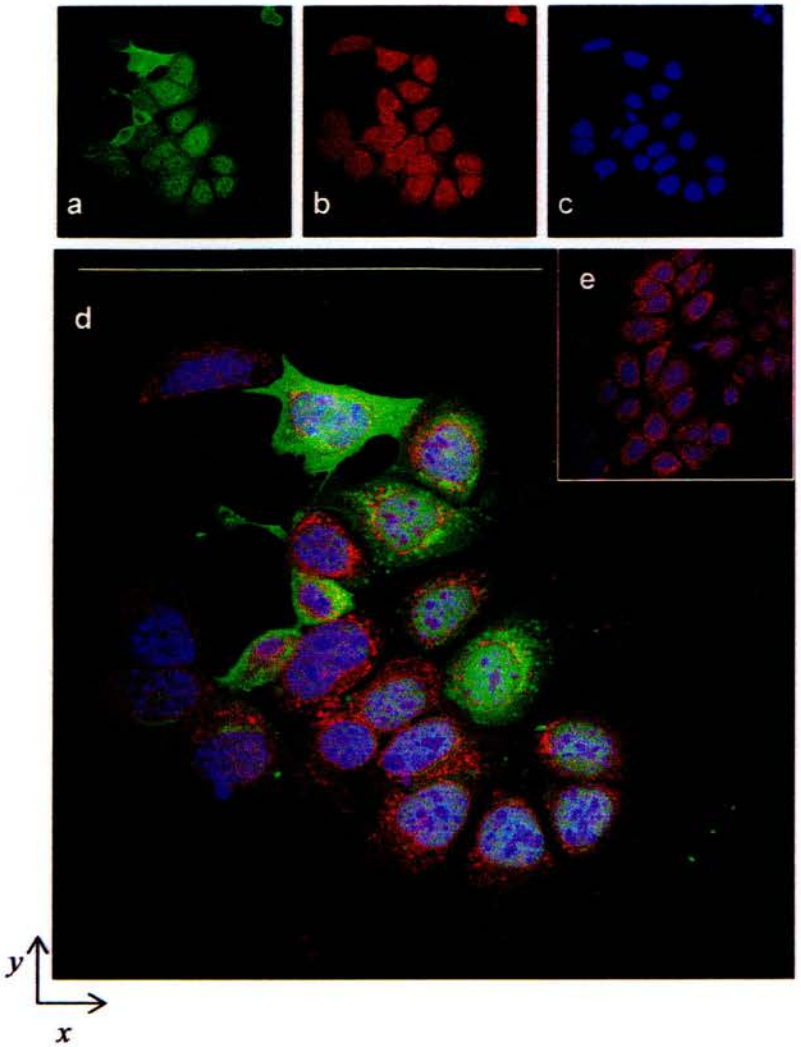


Figure 3.3: DBA binding in mIMCD$_3$ cells, terminally differentiated cells of the adult collecting duct.

Cells were cultured and triple stained with FITC-DBA (a) and TRITC-anti-laminin (b) and topro3 (c).The overlay is also presented (d). DBA bound to various degrees in all cells. A control experiment was performed in parallel without FITC-DBA (e). No staining was seen in the control. These images represent single optical confocal sections. The axes of the image represent the x, y direction. Scale bar =100μm

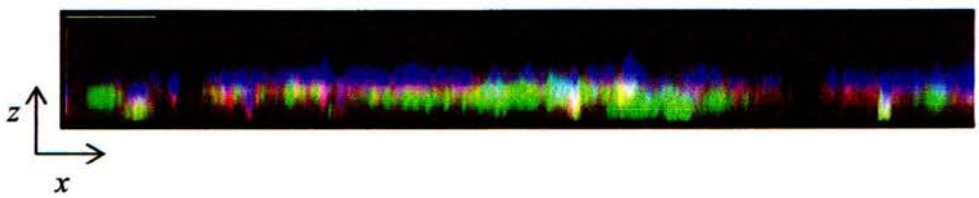


Figure 3.4: 3D image of DBA binding in mIMCD$_3$ cells
Cultured mIMCD$_3$, were triple stained with FITC-DBA and TRITC-anti-laminin and topro3. The image is a 3D representation of a serial section with step size of 3μm which was constructed using Leica confocal software. DBA and anti-laminin bind to the baso-lateral aspect of the cell (it is unclear whether this staining is cytoplasmic or membrane associated). The axes of the image represent the x, z direction. Scale bar = 100μm

3.2.2 *Dolichos biflorus* agglutinin binding with the developing metanephros

Time course analysis of the binding pattern of DBA within the developing metanephros was carried out in order to characterise the precise pattern of DBA binding in the kidney (Michael 2003). Kidneys were dissected at E11.5, by which time they had undergone one round of branching morphogenesis and were at the T-shape stage. They were set up in organ culture for varying lengths of time before staining with FITC-DBA and anti-laminin antibodies (figure 3.5 and figure 3.6). As early as E11.5, DBA binding was found to be restricted to the stalk regions of the ureteric bud and binding was absent from the tip regions (figure 3.5a and b). Anti-laminin staining showed the entire basement membrane of the ureteric bud, indicating the location and presence of the tip regions, continuous with the stalks. A similar binding pattern is seen in metanephric kidneys that were cultured for 24hr, 48hr, 72hr and 96hr further (figure 3.5c-f and figure 3.6a-d). The ureteric bud underwent repetitive rounds of branching morphogenesis, which resulted in an increase in the number of tips and branches and a lengthening of the stalk regions. DBA did not bind to the regions of the tips but strong binding was still seen in the stalk regions. DBA also began to show up in the nephrons which were beginning to form in the surrounding metanephric mesenchyme (figure 3.6c, d).

A feature of branching in many organs is the formation of clefts in the middle of tip ampullae. During branching, a cleft develops in the middle of the expanding tip ampulla during lung development (Moore *et al.* 2005) and salivary gland development (Sakai *et al.* 2003). Similar cleft formations occur during kidney development also (Meyer *et al.* 2004). Higher magnification imaging of cultured kidneys showed that DBA bound to the epithelium under the forming 'cleft' during branching of the tip region (figure 3.6e and f). This rapid up-regulation of DBA ligand expression suggests that the newly forming epithelium, joining the daughter branches, takes on a stalk-like character rapidly during the development of daughter tips.

The binding pattern of DBA was also investigated in kidneys cultured for 144hr (figure 3.7). In more proximal (mature) stalk regions DBA appeared to bind to the basal side of the epithelium with no binding seen apico-laterally. There was co-localisation with laminin in places along the basement membrane. However the

resolution of a confocal microscope (~0.5μm) is not sufficient to separate staining of the basement membrane and the overlying cell membrane (~0.05μm). After 144hr of culture, nephron development had advanced and maturing tubules were obvious. DBA bound very weakly to nephrons (figure3.7d-f). DBA also bound well to the connecting duct regions of the developing collecting duct system. The binding pattern in the connecting ducts and in more distal stalk regions was less uniform with patchy staining seen apically as well as basolaterally (figure 3.7d).

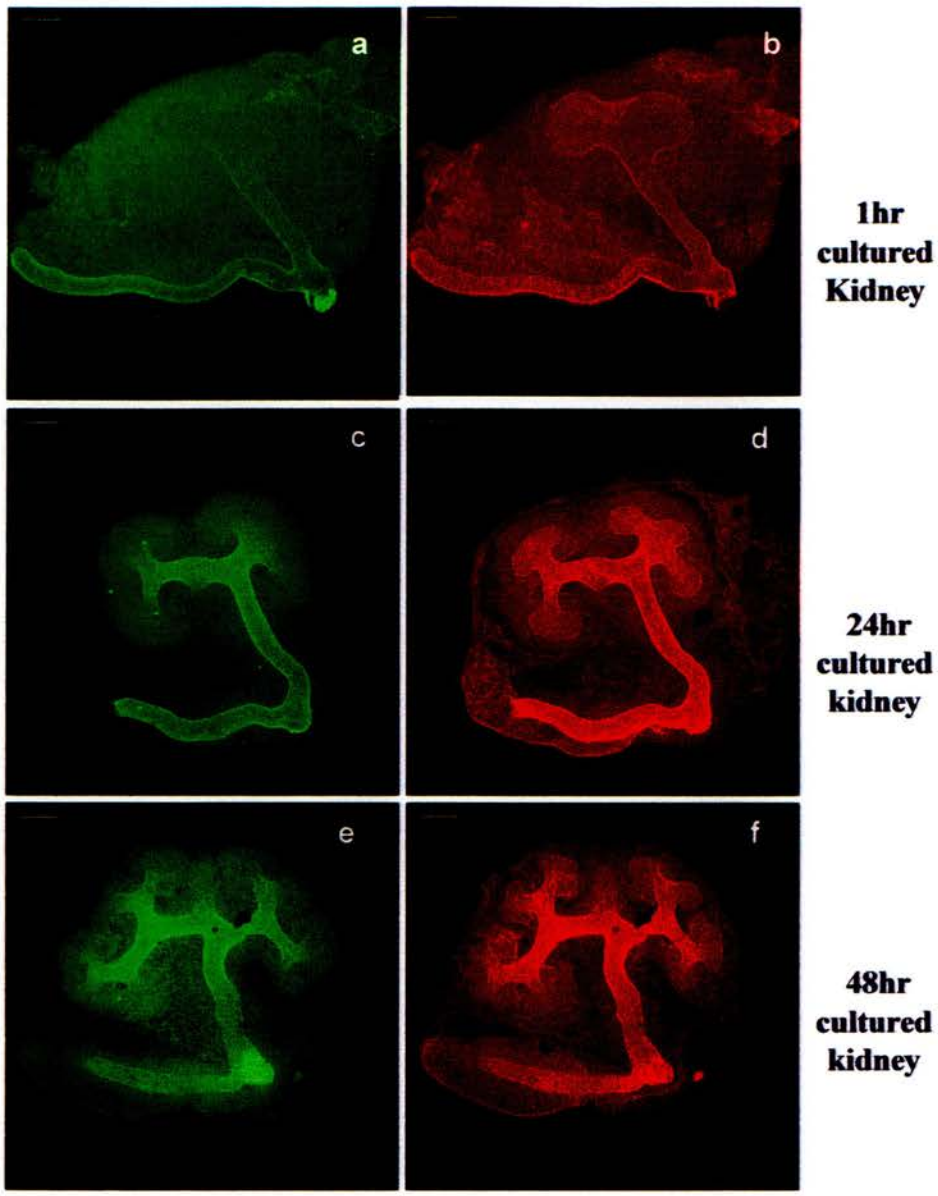


Figure 3.5: DBA binding in kidneys cultured for up to 48hr

Kidneys were double stained with FITC-DBA and TRITC-anti-laminin. Kidneys were cultured for 1hr (a, b), 24hr (c, d), 48hr (e, f). DBA bound specifically to the stalk regions of the ureteric bud. Staining was absent in the tip regions. Scale bar =100μm.

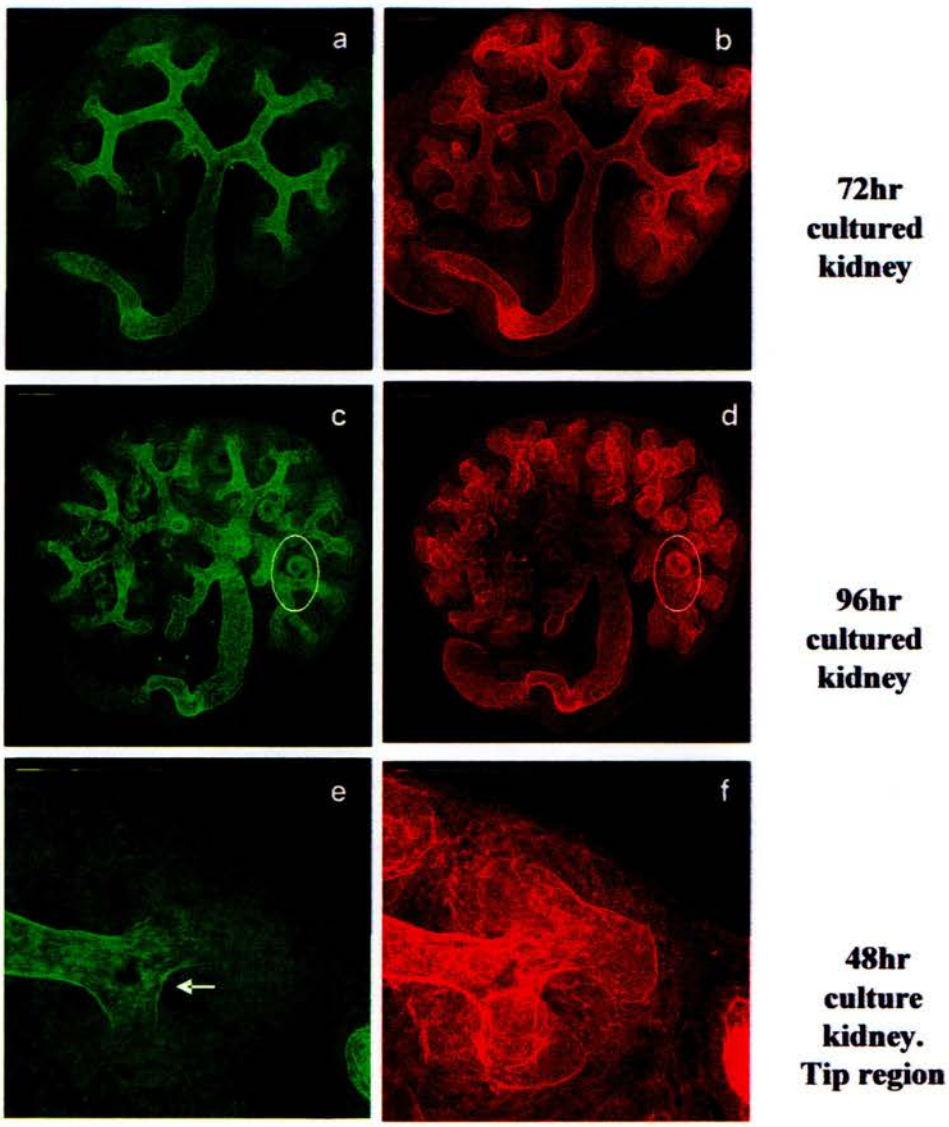


Figure 3.6: DBA binding in kidneys cultured for up to 96hr
Kidneys were double stained with FITC-DBA and TRITC-anti-laminin. Kidneys were cultured for 76hr (a, b) and 96hr (c, d). DBA continued to bind specifically to stalk regions of the ureteric bud. As nephrons formed DBA binding was seen in s-shaped bodies (circle). Tips of a 48hr cultured kidney (e, f) show DBA staining was absent in the tip regions while binding became established in the cleft region (arrow). Scale bar =100μm

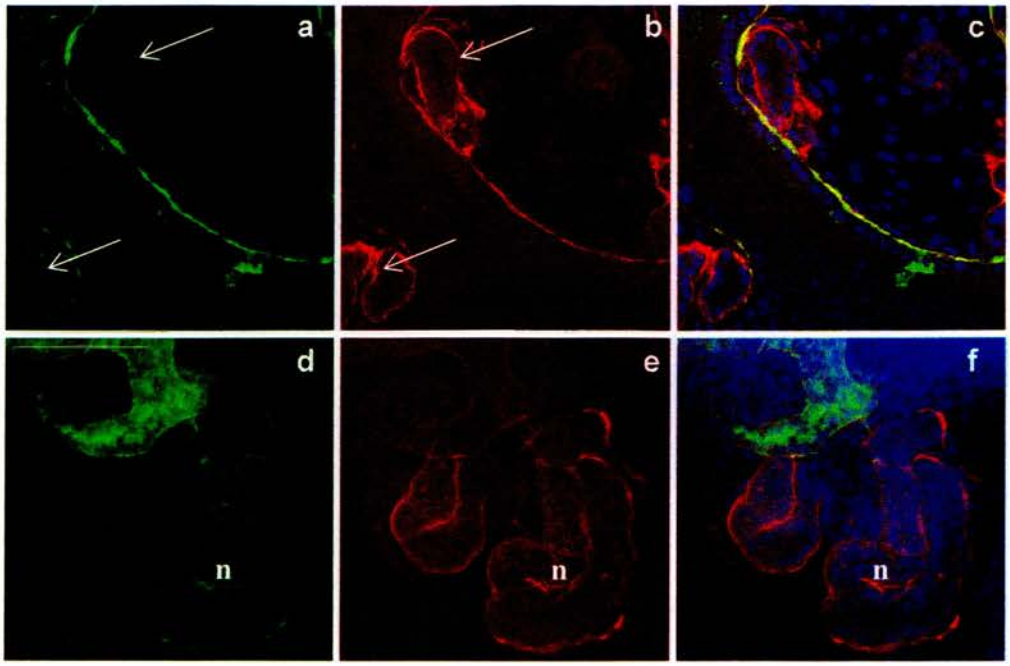


Figure 3.7: High magnification of DBA binding pattern in kidneys cultured for 144hr

Kidneys were cultured for 144hr and stained with FITC-DBA (a, d) and TRITC-anti-laminin (b, e) and topro3. The stalk regions (a, b, c) and developing nephron/connecting ducts (d, e, f) were imaged. In the stalk region DBA bound to the basal side of the ureteric epithelium (a) with little apical or lateral staining. There was some co-localisation with anti-laminin (c) and faint DBA binding in the developing nephrons (arrow). In the connecting ducts, DBA appeared to have a more distributed staining pattern with obvious binding both basally and apico-laterally (d). The neighbouring developing nephron (n) bound DBA weakly. Images a, b, c are single optical sections while images, d, e, f represent a maximum projection of a series of optical sections. Scale bar =100μm

3.2.3 The effect of DBA on branching morphogenesis

It is possible that the sugar residues recognised by DBA play an important role in the kidney. To elucidate the effects of DBA on branching morphogenesis, kidneys were grown for 24hr with or without DBA (10μg/ml). Kidneys were fixed and stained with anti-calbindin-D_{28k}. After 24hr, there was no difference in the number of tips produced per kidney when cultured with or without DBA (figure 3.8). Similarly, when kidneys were removed to fresh medium for 72hr following culture for 24hr with/without DBA, there was no difference in the number of tips per kidney (figure 3.8). Therefore DBA does not have an effect on branching morphogenesis.

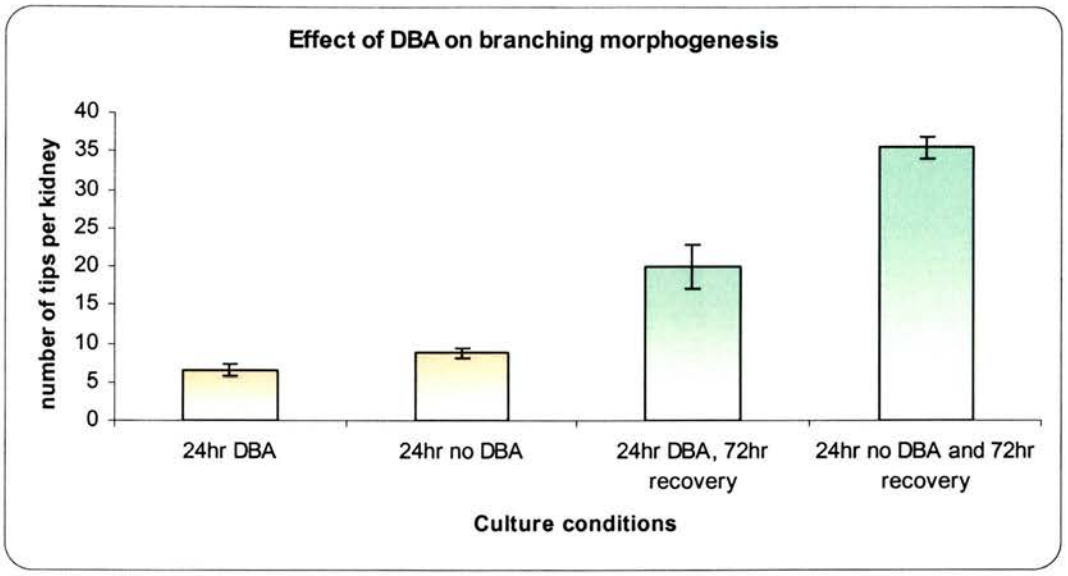


Figure 3.8: Effects of DBA on branching morphogenesis

Tip number per kidney was compared for kidneys grown in organ culture $^{+}$/- 10μg/ml DBA for 24hr and those grown $^{+}$/- 10μg/ml DBA for 24hr with 72hr further culture in fresh medium. The bars represent the mean number of tips per kidney for untransformed data. There were no significant differences between groups 24hr +DBA and 24hr -DBA or groups 24hr +DBA 72hr culture and 24hr -DBA 72hr culture ($F_{3,64}$=280.20, p<0.001). The square root transformation was used for statistical analysis in order to meet the assumptions of normality and homogeneity of variances. Bars = mean of 16 kidneys $^{+}$/- SEM.

3.2.4 Characterisation of *Wnt11* expression by *in situ* hybridisation

The experiments that follow make use of DBA as a specific marker of stalks and require a similarly selective marker of tips. *Wnt11* was chosen as a marker of tip regions (Christiansen *et al.* 1995; Christiansen *et al.* 1996).

To test the suitability of *Wnt11* as a marker of tips;

1. Its expression was characterised by in situ hybridisation to ensure it was expressed solely by the tips of the ureteric bud.
2. It was investigated whether DBA and *Wnt11* observe distinct domains of binding/expression. It was important that *Wnt11* expression respects a boundary with DBA binding in the ureteric bud. *Wnt11* could not be a suitable marker of tip regions if it had an overlapping expression domain with the binding domain of DBA.

Embryo whole-mount *in situ* hybridisation was carried out to test the specificity of the *Wnt11 in situ* hybridisation probe. Whole-mount embryo *in situ* hybridisation for *Wnt11* at E11.5 revealed an expression pattern as previously described (Christiansen *et al.* 1995; Christiansen *et al.* 1996) (figure 3.9a, c). Expression was detected in the otic placodes, somites, and the upper border of the maxillary prominence and was also detected to some extent in the developing hindbrain and forebrain. Expression was also detected in the central area of the paddle shaped forelimb and hindlimb plates. Little expression was detected with the sense probe although some staining is seen in the developing brain ventricles and also around the extremities of the tail and limbs. This staining is assumed to be due to probe trapping and residual staining (figure 3.9b, d).

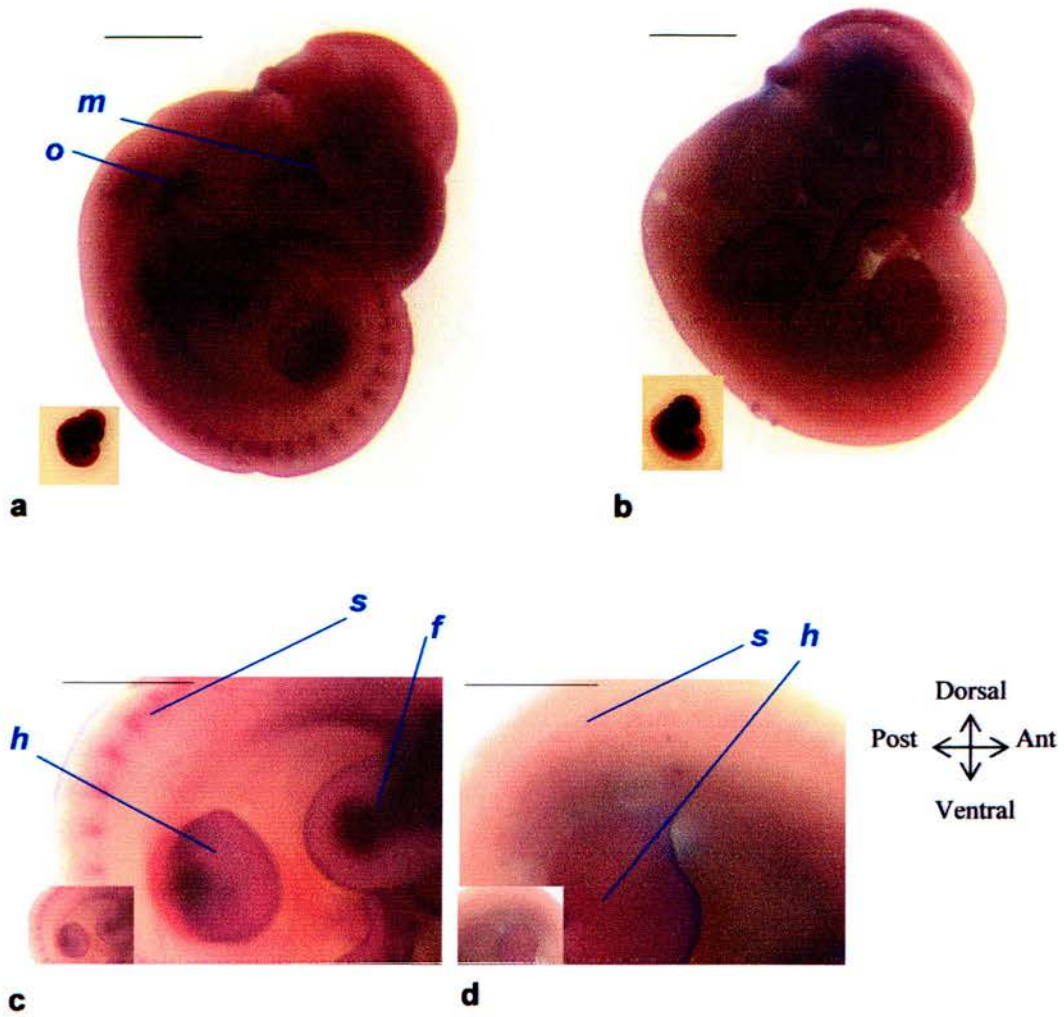


Figure 3.9: Whole-mount *Wnt11 insitu* hybridisation on E11.5 embryos.

E11.5 embryos were processed for sense (a, c) and antisense (b, d) *Wnt11* in situ hybridisation. Antisense staining was obvious in the somites (s), hindlimb (h), forelimb (f), otic vesicle (o) and maxillary prominence (m). Little staining was seen with the sense probe although some residual staining was evident at the edges of the tail and limbs and some probe trapping was seen in the ventricles. The inserts show the image prior to processing using Adobe Photoshop. Image levels were adjusted and brightness was increased equally for all images using Adobe Photoshop. Scale bar =1000μm.

3.2.5 *Wnt11* is expressed specifically in the tips of the ureteric bud

The specific nature of *Wnt11* expression had to be characterised further to ensure that expression was limited to the tip regions as early as E11.5, the stage at which dissections were carried out (figure 3.10). *Wnt11*, a gene previously reported to be expressed by the tips of the ureteric bud from as early as E11.5 (Kispert *et al.* 1996; Majumdar *et al.* 2003) was characterised using embryos and cultured kidneys for its usefulness as a marker of tip regions.

In E14.5 kidneys *Wnt11* expression was detected only in the ampullae of the ureteric bud, which at this stage were located at the periphery of the organ. Expression was absent from the developing ureter and the metanephric mesenchyme (as well as its nephron and stromal derivatives) (figure 3.10b). Staining was absent from the surrounding tissues including the gonad and the adrenal glands (figure 3.10a). Control staining, using the *Wnt11* sense probe, resulted in a small amount of residual, non specific staining at the periphery of the kidney (figure 3.10c).

At E11.5, the ureteric bud is T-shaped. It is at this stage that the kidneys used in the majority of my experiments were dissected for culture. Even though only one round of branching morphogenesis had taken place, *Wnt11* expression was restricted to the tip region and was undetectable in the stalk region (figure 3.10d). However some *Wnt11* expression was also detectable in the vicinity of the Wolffian duct.

A similar expression pattern was seen with E11.5 kidneys cultured for 48hr in organ culture (figure 3.10e). Expression was obvious in the multiple tip regions that had developed but was undetectable in the stalk regions extending in-between the tips. Often *Wnt11* expression was detected to a lower degree in the mesenchyme around the Wolffian duct outside the kidney. There was no staining with the sense probe for *Wnt11* (figure 3.10f).

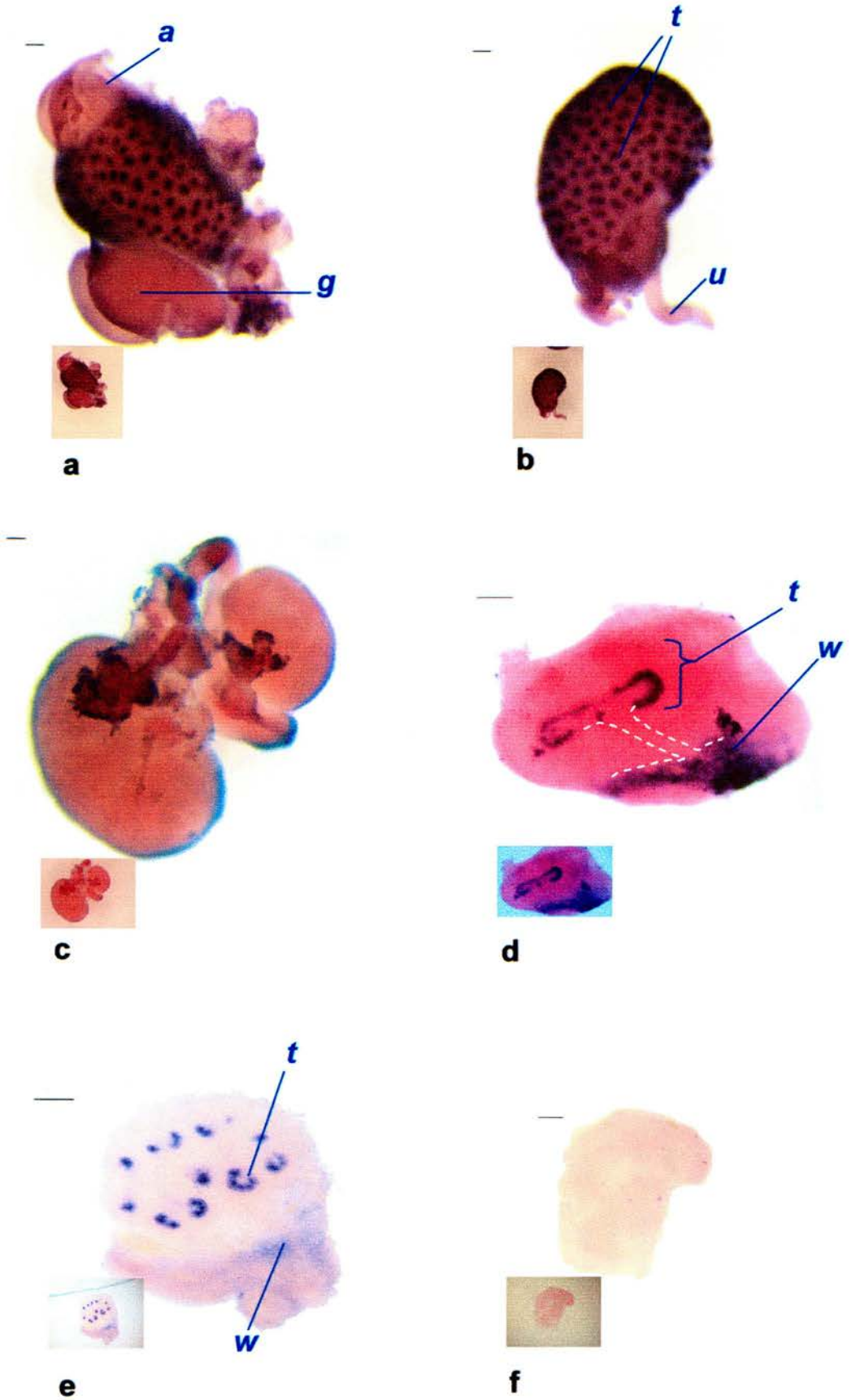


Figure 3.10

Figure 3.10: *Wnt11 insitu* hybridisation on E14.5 kidneys whole-mounts and E11.5 kidneys cultured *ex vivo* for 48hr.

Kidneys and some associated structures were dissected from the urogenital region of E14.5 kidneys (a, b, c). E11.5 kidneys were also removed and processed as wholemounts (d) or cultured for 48hr (e, f). Tissue samples were processed for antisense (a, b, d, e) or sense (c, f) *Wnt11 in situ* hybridisation. In the E14.5 kidney antisense staining (figure a, b) was seen in the tip regions (t) of the ureteric bud as they extended towards the periphery of the E14.5 kidney. Staining is absent from the ureter (u). Expression is absent from the developing adrenal gland (a) and gonad (g). At E11.5 *Wnt11* expression is confined to the tip region (t) and excluded from the stalk (dotted white line). Some staining is seen associated with the Wolffian duct (w). Similarly in the E11.5 kidney culture for 48hr *Wnt11* expression (figure e) is seen in discreet domains corresponding to the tip regions of the ureteric bud (t). Staining is weakly detected around the Wolffian duct as well (w). Little staining is seen with the *Wnt11* sense probe and is assumed to be residual (figure c, e). The inserts show the image prior to processing using Adobe Photoshop. Image levels were adjusted and brightness was increased equally for all images using Adobe Photoshop. Scale bar =100μm.

3.2.6 The position of the DBA binding domain relative to the *Wnt11* expression domain in the ureteric bud

Having established that *Wnt11* is expressed by the ampullae of the ureteric bud and that DBA is expressed proximal to the end of the ureteric bud, I investigated whether they observed a common boundary or whether there was significant overlap in their expression/binding domains. To determine this, the length of the *Wnt11* expression domain and the length of DBA-free ureteric bud were measured. The length of the *Wnt11* expression domain was measured from i*n situ* hybridisation images of E11.5 kidneys cultured for 48hr. The length of the *Wnt11* expression domain varied from 30-110μm (mean length = 70.82μm) (figure 3.11). The length of DBA-free ureteric bud was taken as the distance from the terminal ends to the DBA binding region of the ureteric bud. It appeared that DBA bound to the ureteric bud between 30-120μm from the edge of the tips (mean length = 66.52μm) (figure 3.12). When the length of the *Wnt11* expression domain and the length of DBA-free ureteric bud were compared there was no statistically significant difference between the two lengths (figure 3.13). This suggested that there was little overlap between the domain of *Wnt11* expression and the domain of DBA binding within the ureteric bud.

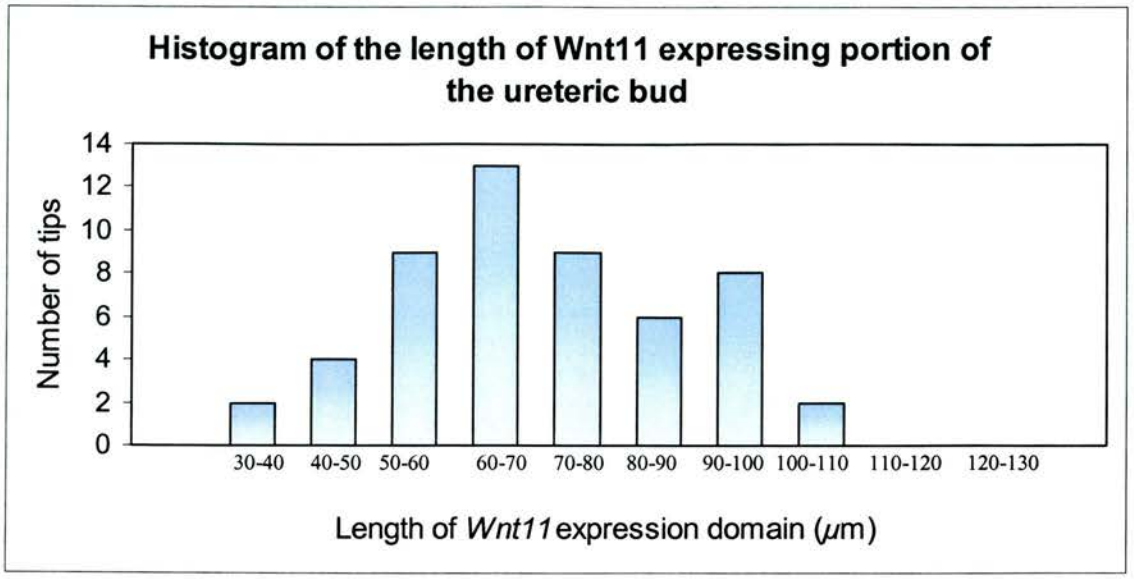


Figure 3.11: Histogram of the length of the *Wnt11* expression domain of the ureteric bud.

The length of the *Wnt11* expression portion of the ureteric bud E11.5 +48hr cultured kidneys (n=53 tips) was estimated from *Wnt11 in situ* hybridisation images.

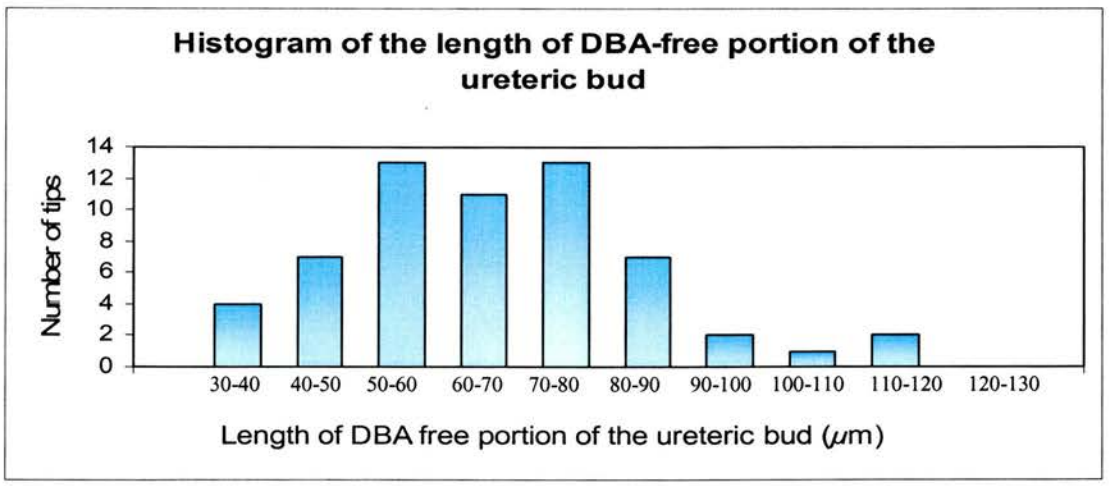


Figure 3.12: Histogram of the length of the DBA-free ureteric bud.

The length of the DBA-free portion of the ureteric bud of E11.5 + 48hr cultured kidneys (n= 60 tips) was estimated by measuring along the axis of the branch, from the terminal edge to the stalk region (as defined by DBA binding).

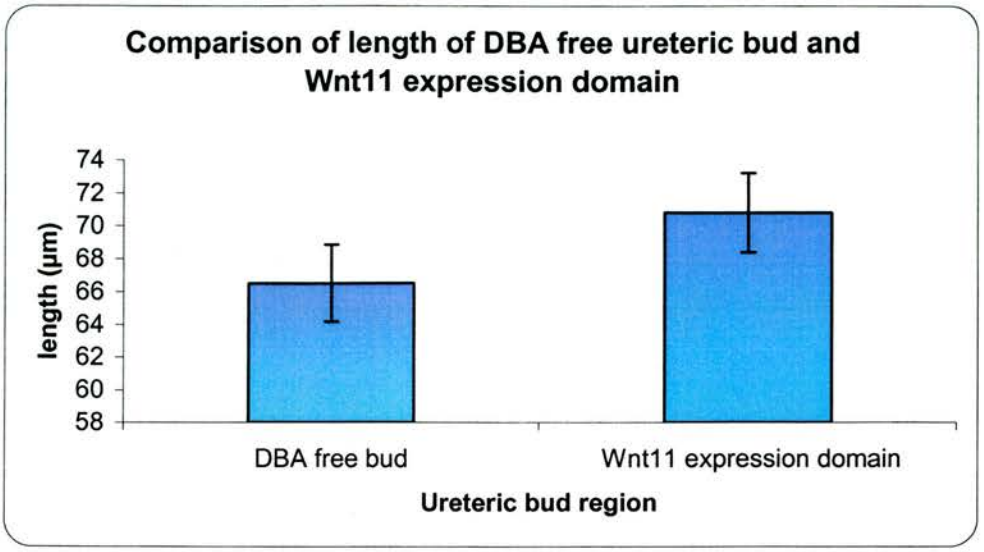


Figure 3.13: Comparison of the length of the DBA free region of the ureteric bud with the length of the *Wnt11* expressing region.

The average length of DBA free region was compared to the average length of the *Wnt11* expressing region of the ureteric bud of 48hr cultured kidneys. The bars represent the mean length of untransformed data. There were no significant differences between the lengths of the two ureteric bud regions ($F_{1,111}$=1.72, p=0.193). The square root transformation was used for statistical analysis in order to meet the assumptions of normality and homogeneity of variances. Bars = mean of 60 kidneys $^{+}$/- SEM.

3.2.7 Blocking branching morphogenesis induces a change in cell identity in the tips of the ureteric bud

Branching morphogenesis can be halted in various ways in culture e.g. by the addition of function blocking antibodies to GDNF (Fisher *et al.* 2001; Davies *et al.* 1999; Vega *et al.* 1996), yet little is known of what happens to cell identity within the ureteric bud when branching morphogenesis is inactivated. 30mM sodium chlorate, as previously described, is an effective inhibitor of kidney branching morphogenesis (Davies *et al.* 1995; Kispert *et al.* 1996). Using DBA as a marker of stalk regions and *Wnt11* as a marker of tip regions, the hypothesis that tip regions of the ureteric bud become stalk–like when branching morphogenesis is inhibited was tested. Cell identity within the ureteric bud was investigated when branching was

inhibited in kidneys cultured in the presence of sodium chlorate (figure 3.14). Affected kidneys showed a stunted morphology; with the ureteric bud remaining T-shaped, whereas control kidneys had undergone a few rounds of branching morphogenesis. The cessation of branching coincided with a dramatic change in the binding pattern of DBA which now bound to the entire ureteric bud epithelium (compare figure 3.14a and d).

It had been previously reported that when kidneys are grown in 30mM sodium chlorate, there is an obvious loss of the tip marker *Wnt11* from the ureteric bud (Kispert *et al.* 1996). Therefore, i*n situ* hybridisation for *Wnt11* expression was also performed on kidneys cultured with or without chlorate to show that the loss of *Wnt11,* as Kispert described, is concomitant with the increase in DBA binding to the entire ureteric bud (compare figure 3.14c and f).

In summary, it seems that DBA binds to tip regions of the ureteric epithelium when branching morphogenesis is inactivated. When branching morphogenesis is blocked in kidneys there is a striking change in cell behaviour of the tips of the ureteric bud. The tip regions appear to transform to a stalk cell identity by down-regulating the expression of tip markers, such as *Wnt11*, and up-regulating stalk markers, such as the expression of the glycoprotein to which DBA binds.

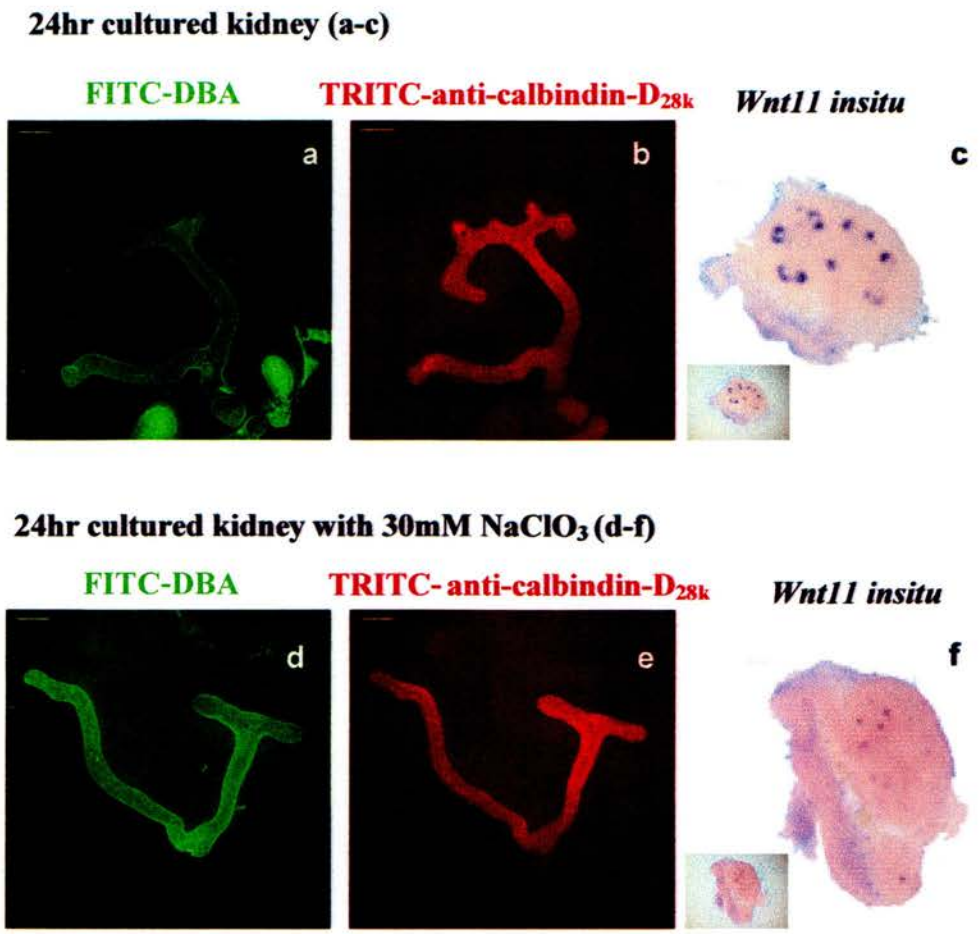


Figure 3.14: DBA binding in kidneys cultured for up to 24hr with or without 30mM $NaClO_3$

Kidneys were double stained with FITC-DBA (a, d) and TRITC-anti-calbindin-D_{28k} (b, e). *Wnt11* expression was detected by insitu hybridisation (c, f). Kidneys were cultured with culture medium for 24hr (a, b, c) or with culture medium supplemented with 30mM $NaClO_3$ for 24hr (d, e, f). DBA bound specifically to the stalk regions of the ureteric bud while *Wnt11* was expressed by cells of the tips when kidneys were grown in culture medium. Supplementation of the culture medium with $NaClO_3$ caused DBA to stain the tip regions as well and *Wnt11* expression was downregulated. Inserts in c and f show the images prior to image processing using Adobe Photoshop. Image procession was carried out on image c and f to remove the filter on which the kidney was cultured from the image. Scale bar =100μm.

3.2.8 The change in cell identity, as induced by chlorate, is reversible

The effects of chlorate on branching morphogenesis and cell identity are quite dramatic. In order to investigate whether the change in cell behaviour is reversible, kidneys were cultured for 24hr in chlorate and then removed to chlorate-free medium to recover for periods of 24hr, 48hr and 72hr (figure 3.15, 3.16 and 3.17). After only 24hr of recovery, branching morphogenesis began to occur again and the expression of the tip marker *Wnt11* returned. At the same time DBA binding decreased in the tip regions. This progression was more obvious after 48hr and 72hr of culture. Anti-laminin staining revealed that the ureteric bud induced nephrogenesis in the surrounding mesenchyme even though it had been treated with chlorate. This confirms that chlorate, although profoundly affecting branching behaviour of the ureteric bud, has little effect on nephrogenesis (Davies *et al.* 1995). Overall it seems that the effects of culturing kidneys with 30mM chlorate are reversible both with regard to the retardation of branching and the change in tip cell identity.

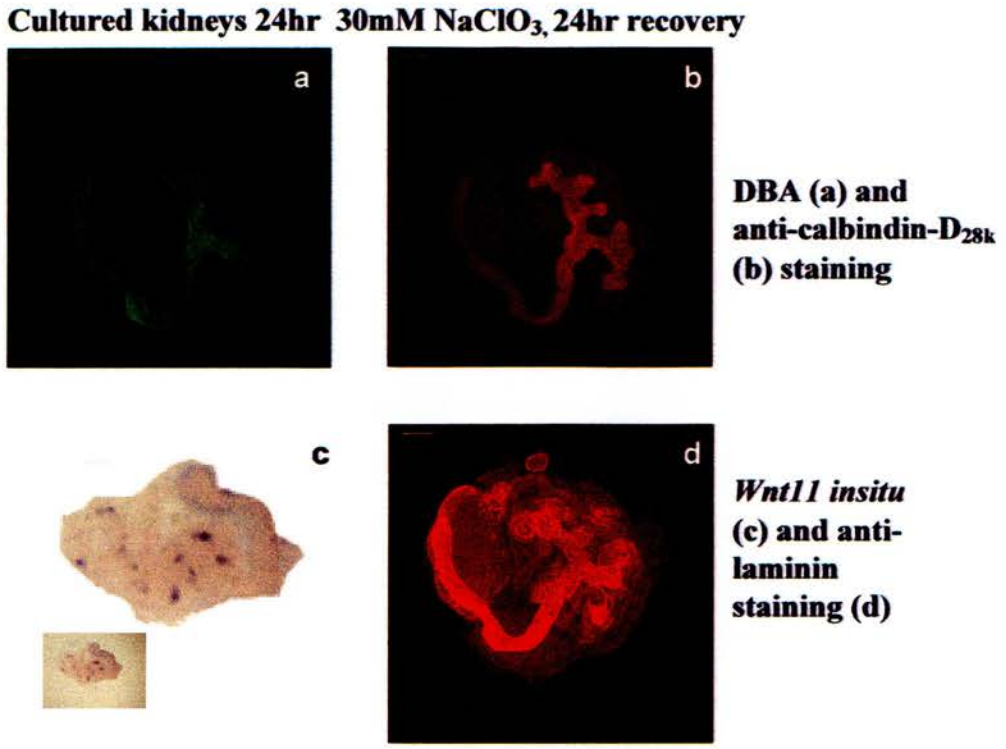


Figure 3.15: DBA binding in kidneys recovering for 24hr from culture with 30mM $NaClO_3$.

Kidneys were double stained with FITC-DBA (a) and TRITC-anti-calbindin-D_{28k} (b). Others were processed for *Wnt11* insitu hybridisation (c) or stained for anti-laminin (d). Kidneys were cultured with culture medium supplemented with 30mM $NaClO_3$ for 24hr and transferred to culture medium for further 24hr culture. After 24hr of recovery DBA highlighted the stalk regions but avoided binding to the tip regions. *Wnt11* expression returned but remained confined to the tips of the ureteric bud (c). Nephrogenesis had taken place as evidenced by the s-shaped bodies in d. The insert in c shows the image prior to processing using Adobe Photoshop. Image procession was carried out on image c to remove the filter on which the kidney was cultured from the image. Scale bar =100μm.

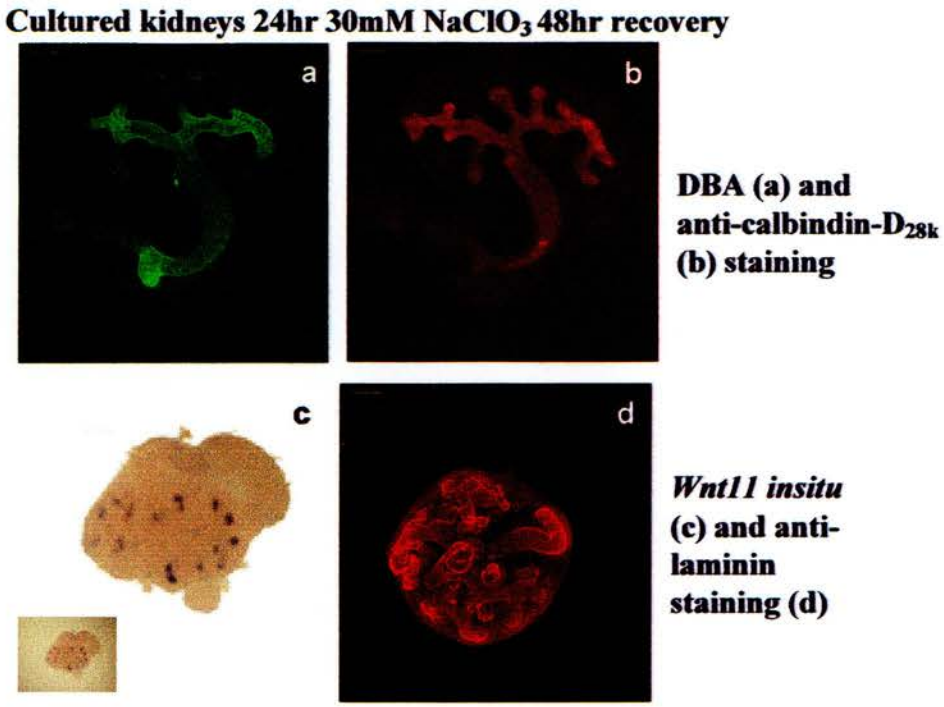
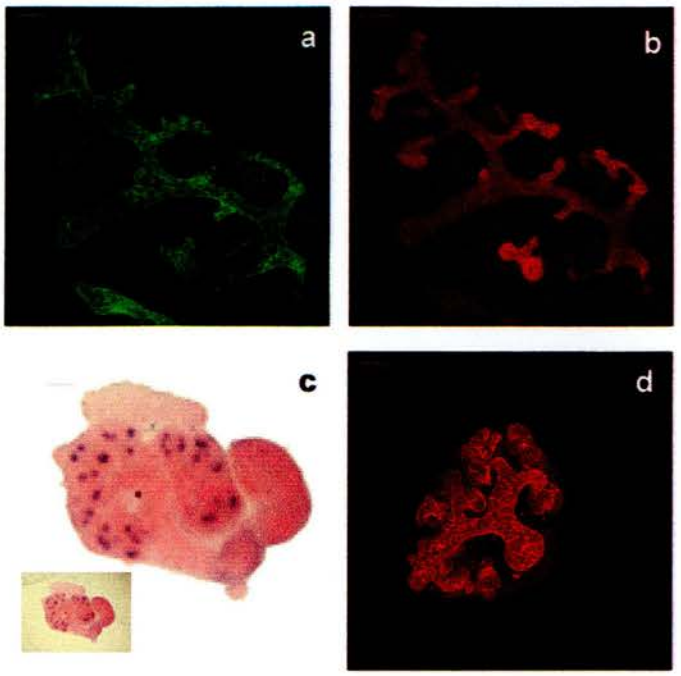


Figure 3.16: DBA binding in kidneys recovering for 48hr from culture with 30mM $NaClO_3$.

Kidneys were double stained with FITC-DBA (a) and TRITC-anti-calbindin-D_{28k} (b). Some kidneys were processed for *Wnt11* insitu hybridisation (c) while others were stained for anti-laminin (d). Kidneys were cultured with culture medium supplemented with 30mM $NaClO_3$ for 24hr and transferred to culture medium for a further 48hr of culture. After a recovery period of 48hr DBA highlighted the stalk regions but avoided the tip regions. *Wnt11* expression was returning but remained confined to the tips of the ureteric bud (c). Nephrogenesis had taken place as s-shaped bodies are seen in d. The insert in c shows the image prior to processing using Adobe Photoshop. Image procession was carried out on image c to remove the filter on which the kidney was cultured from the image. Scale bar =100μm.

Cultured kidneys 24hr 30mM $NaClO_3$, 72hr recovery



DBA (a) and anti-calbindin-D_{28k} (b) staining

***Wnt11 insitu* (c) and anti-laminin staining (d)**

Figure 3.17: DBA binding in kidneys recovering for 72hr from culture with 30mM $NaClO_3$.

Kidneys were double stained with FITC-DBA (a) and TRITC-anti-calbindin-D_{28k} (b). Some kidneys were stained for anti-laminin (d) and others were processed for *Wnt11* insitu hybridisation (c). All kidneys were cultured with culture medium supplemented with 30mM $NaClO_3$ for 24hr and were then transferred to culture medium for a further 72hr of culture. A 72hr recovery period re-established DBA staining of the stalks and not of the tips. *Wnt11* expression by the tips also returned(c). Nephrogenesis occurred vigorously as numerous s-shaped bodies were seen in d. Insert in C shows image prior to image processing using Adobe Photoshop. Image procession was carried out on image c to remove the filter on which the kidney was cultured from the image. Scale bar =100μm.

3.3 Discussion

The ureteric bud epithelium is composed of at least two different cell types which can be categorised based on their location within the epithelium (either at the tips or stalks of the epithelium). This categorisation based on location coincides with a particular branching behaviour with the cells of tips giving rise to branches while those of the stalks rarely give rise to new branches (Watanabe *et al.* 2004). DBA is introduced as a novel marker of stalks of the ureteric bud.

Statistically there was no difference in the length of the DBA free region of the ureteric bud and the length of the *Wnt11* expression domain so there is not likely to be an overlap in the DBA binding domain and the *Wnt11* expression domain of the ureteric bud. Although it does not confirm that the two domains of binding/expression are mutually exclusive these data does support such a layout. Previous work in the lab estimated the rate of proliferation in the ureteric bud by BrdU incorporation assays (Michael *et al.* 2004). Cell proliferation was found to be localised to the tips of the bud and was relatively low in the stalk regions (figure 3.18) (Michael *et al.* 2004).

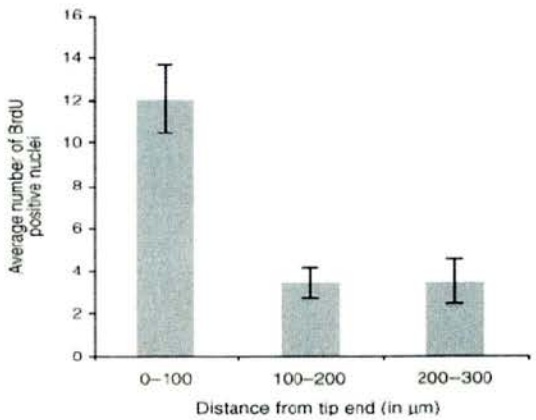


Figure 3.18: Cell proliferation is localised to the tips of the ureteric bud during branching morphogenesis.

BrdU (100μM) was incorporated for the last 16 hr of 32 hr culture of E11.5 kidneys. BrdU positive nuclei were counted along the ureteric bud at 100μm distance from the tip edge towards the stalk (0-100μm = tip, 100-200μm and 200-300μm =stalk. Error bars represent ± standard error of the mean. This figure is reproduced with permission, from Michael *et al.* 2004.

The transition from the *Wnt11* expression domain to the DBA binding region of the ureteric bud occurs between 67-71μm. The proliferation studies carried out by Michael *et al.* concluded that cell proliferation is relatively higher within the first 100μm from the edge of the ureteric bud (a higher level of spatial resolution was not carried out in this study). Therefore it is plausible that the stalk regions of the ureteric bud become established within the region where cell proliferation is the highest (figure 3.19). Further analysis of cell proliferation may reveal that the zone of highly proliferating cells is in fact more restricted towards the edge of the tip than 100μm, perhaps remaining within the *Wnt11* expression domain and not within the DBA binding domain (figure 3.19).

The length of the DBA free region of the ureteric bud was measured from lectin histochemistry images while the length of the *Wnt11* expression domain of the ureteric bud was measured from *insitu* hybridisation images. The fixation processes for the lectin histochemistry technique and the *insitu* hybridisation technique differed slightly (refer to pg. 55 and 63) as did the storage times for the various samples. It is unclear if the variability in experimental procedures affects the explant size itself but it can not be discounted. Should the lectin histochemistry and *insitu* hybridisation techniques result in differentially sized kidney explants then the measurements of tip regions using these two techniques would not be absolutely comparable. To overcome this possible limitation the length of the tip region should be related to either the overall size of the explant itself or the length of the ureteric bud tree. Such a measurement would overcome the possible variability in tip lengths introduced due to the different experimental techniques used. Although there is no suitable Wnt11antibody for immunohistochemistry it would be optimal to carry out double staining for DBA and *Wnt11* to confirm whether their expression/binding domains are mutually exclusive.

DBA has previously been used as a marker of terminally differentiated cell types such as keratinocytes (Hrdlickova-Cela *et al.* 2001; Dvorankova *et al.* 2002). The idea of DBA as a marker of differentiated cells of the ureteric bud is raised based on the fact that DBA has affinity for relatively quiescent cells, the cells of the stalk. The proliferation zone of the ureteric bud is found within the first 100μm from the edge of the tip towards the stalk (Michael *et al.* 2004) and it is within this region

that the stalk becomes established. The concentration of proliferating cells at the tips of the ureteric epithelium (Michael *et al.* 2004) and the observed cell movements within the bud itself (Shakya *et al.* 2005a) suggest a model in which stalk cells arise from tip cells. Whether the transition from tip cells to stalk cells is an irreversible differentiation process will be investigated elsewhere (see chapter 4).

The ureteric bud has been shown previously to respond and branch well when embedded in heterologous mesenchyme and can alter its cell identity to those of other epithelial organs. For example, the ureteric bud expresses the type II pneumocyte marker SP-C when recombined with lung mesenchyme (Lin *et al.* 2001; Vainio *et al.* 2003). However little is known of the changes of cell behaviour under active and inactive branching within its own mesenchyme. Although the ureteric bud cells appear differentiated into tip and stalk cells they retain a similar compliance and ability to change their branching behaviour and cell identity. This implies that the transition from tips to stalks is not an irreversible differentiation but that the tip or stalk cells of the ureteric bud can change to the identity of the other.

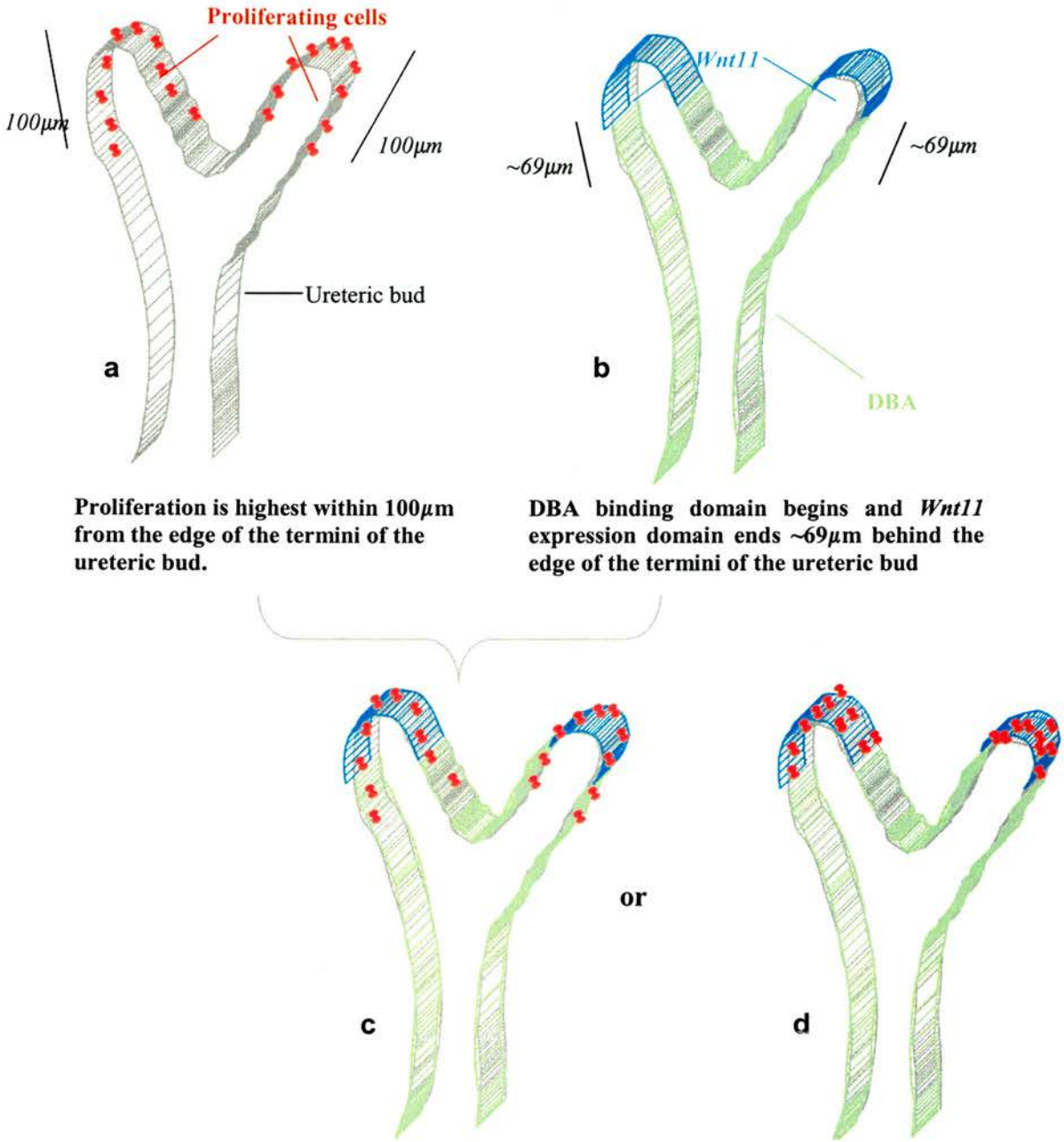


Figure 3.19: The position of DBA-free regions of the ureteric bud relative to the areas of highest proliferation

Michael *et al.* demonstrated that the initial 100μm from the edge of a tip corresponded to the area of highest proliferation (a) (Michael *et al.* 2004). The resolution of this study was limited to 100μm and did not investigate whether proliferation was restricted further towards the edge of the tip. Measurement of the length of DBA-free ureteric bud and the *Wnt11* expression domain is ~69μm (b). Therefore the transition from *Wnt11* expressing region to DBA binding region of the ureteric bud is located within the regions where cell proliferation is highest (c). If proliferation is restricted within a smaller distance from the edge of the tip, it is also possible that the DBA-free region/*Wnt11* expressing region of the ureteric bud corresponds to the region of high cell proliferation (d).

In culture it appears that the tips, the actively branching regions of the epithelium, do not have any affinity for DBA. It has been reported that DBA binds to the collecting duct ampulla of the neonate rabbit (Kispert *et al.* 1996; Schumacher *et al.* 2002) suggesting that DBA binding is up regulated when development is nearing completion. It is possible that the expression of the DBA ligand is involved in the natural cessation of branching morphogenesis as well as the induced cessation as seen with the addition of chlorate or anti-GDNF antibodies. However it may be the case that the DBA ligand is borne on a different molecule during later stages of collecting duct development.

Holthofer *et al.* carried out studies on DBA binding in kidneys along with staining for markers of principle cells (Na^{2+}/K^{+} ATPase) and intercalated cells (carbonic anhydrase II). They suggested that DBA bound mainly to principle cells (Holthofer *et al.* 1988; Holthofer 1988) but also reported that DBA had an affinity for a small proportion of intercalated cells of the adult collecting duct (Holthofer *et al.* 1987). Binding of DBA takes places specifically at the apical domain of the cell membrane in both cell types (Holthofer *et al.* 1987). As we have confirmed, DBA binds quite well to $mIMCD_3$ cells, which are thought to be differentiated cells of the adult collecting duct. In $mIMCD_3$ cells DBA does not bind to the apical cell domain (either intracellularly or at the cell membrane) but a second cell line, MDCK cells, often used as a model of branching morphogenesis, show strict binding of DBA to the apical domain of the cell clearly separated from the laminin rich basal domain (Bao *et al.* 1999). Studies on collecting ducts suggest that DBA binds preferentially to the apices of cells (Holthofer *et al.* 1987). The differences in the binding patterns between $mIMCD_3$ cells and the collecting duct studies may be due to heterogeneity of cell composition of the collecting duct segments (*i.e.* they are cortical collecting ducts instead of inner medullary collecting ducts). The $mIMCD_3$ cells may establish apical polarisation of the DBA ligand in response to the environment. Other receptor components such as the γ-subunit of Na-K-ATPase are incorporated into plasma membranes of $mIMCD_3$ cells in response to hypertonicity (Pihakaski-Maunsbach *et al.* 2005).

During development DBA binds to the basal side of proximal (more mature) stalk regions and shows a patchy apico-basolateral staining in the distal stalks as well as in

the connecting ducts; all regions in which branching morphogenesis is rare. If the apical binding pattern of DBA seen in adult collecting ducts is reflective of the normal collecting duct cell binding pattern then there must be a shift in the binding pattern of DBA from the basal domain of the maturing stalks to the apical binding seen with adult collecting ducts. This suggests perhaps, that the developing stalks of the ureteric bud are not yet correctly polarised. It may be important to consider that the cells of the stalk regions, which were investigated in my experiments, may correspond to the developing transitional epithelium instead of the collecting ducts. It is also possible that the sugar group to which DBA binds is a modification of two different proteins expressed in older and younger kidneys.

The application of GDNF to metanephric organ culture increases cell proliferation (Michael *et al.* 2004) in parallel with a decrease in DBA binding. As branch formation seems to be coupled to the reduction in DBA binding it is tempting to speculate that the glycoprotein to which DBA is binding has a role in suppressing aberrant branch formation. I did not find any effect of DBA on ureteric bud branching so it is unlikely that the sugar residues to which DBA binds have active functions in branching morphogenesis. The ability of the ureteric epithelium to change its binding affinity for DBA easily and transiently suggests that the molecule to which DBA binds, can be quickly synthesised and cleared away in response to branching stimuli. Unfortunately the substance, to which DBA binds within the kidney, is as yet unknown. We can not be certain of its origin either, whether synthesised by the ureteric epithelium or the mesenchymal cells, although the high binding of DBA to mIMCD$_3$ cells suggests it probably originates from the epithelium. One putative candidate binding substance for DBA has been suggested (Stuart *et al.* 2003). Embigin is a glycosylated transmembrane glycoprotein and belongs to the immunoglobulin superfamily (Guenette *et al.* 1997; Fan *et al.* 1998; Stuart *et al.* 2003) and is thought to carry developmentally regulated carbohydrate markers (Fan *et al.* 1998). Embigin was initially cloned as a protein carrying the carbohydrate binding epitope for DBA (Ozawa *et al.* 1988; Fan *et al.* 1998). The expression of embigin is similar to the DBA binding pattern during early embryonic development although mRNA for embigin is scarcely detectable from E10.5 onwards by *in situ* hybridisation (Fan *et al.* 1998). Microarray analysis reports high embigin

expression in the isolated ureteric bud epithelium that has been cultured for 5 days (Stuart *et al.* 2003). Direct analysis of DBA binding to embigin in the developing kidney remains to be carried out and as of yet little is known of embigin's role in modulating ureteric bud branching.

Molecular characterisation of DBA receptors in the neonate rabbit kidney has been initiated. SDS-PAGE analysis and 2D electrophoresis has revealed a number of protein spots that showed affinity for DBA (Schumacher *et al.* 2002). The detected spots did not stain with Coomassie blue which suggests they have low protein but high carbohydrate content, as do proteoglycans (Schumacher *et al.* 2002). The DBA binding proteins have not yet been fully characterised although the authors propose to carry out further analysis using mass spectroscopy (Schumacher *et al.* 2002). As of yet it is unclear if there are single or multiple DBA receptor proteins expressed by the developing collecting duct.

Although it is difficult to be certain without time lapse imaging, it seems that the branching behaviour only returns to those areas of the epithelium which were originally tips. It is possible that the inhibited tips somehow 'remember' that they are tips and easily revert to actively branching because of a retained awareness of their 'tipness'. If so, old tips and stalks may be differentiated even when their DBA/*Wnt11* binding/expression patterns are the same. But there is also the question of why the stalks of the ureteric bud do not branch more vigorously when chlorate is removed from kidney cultures. It is possible that the surrounding mesenchyme plays a role in retarding branching in the stalks compared to the tips. The identification of more differentially-expressed markers both for the epithelial and mesenchymal components of the kidneys would shed light on this. For example, it is plausible that 'stop' signals from the stalk associated mesenchyme inhibit branching morphogenesis.

In conclusion DBA has been identified as a novel marker of regions of the ureteric bud in which branching morphogenesis is dormant, whether these regions are stalk regions or tip regions in which branching has been suppressed. Also it has been shown that the distinction between tip and stalk cells may be much more plastic and malleable than had previously been supposed.

Chapter 4

Generation of branching tips from the stalks of the ureteric bud.

4.1 Introduction

The ureteric bud branches in a quite predictable manner. Branching events are mostly dichotomous and occur predominantly at the terminal ends of the branching epithelium with only 6% of branching events arising as a lateral offshoot of the stalks of the ureteric bud of cultured kidneys (Watanabe *et al.* 2004). As presented in the previous chapter, at least two different cell populations (tip and stalk) are present within the ureteric bud and their identity must be tightly regulated if branching morphogenesis is to be correctly spatially regulated.

There are significant differences between tip and stalk regions, both structural (Meyer *et al.* 2004; Michael *et al.* 2005) and in their gene expression patterns (Schmidt-Ott *et al.* 2005; Davies 2005). A substantial array of marker genes for tips and stalks have been identified (Schmidt-Ott *et al.* 2005; Davies 2005) and their importance in regulating kidney development is beginning to be studied. Ultra-structural analyses of the ureteric bud show that the cells of the stalk show a more epithelial like structure as they appear morphologically to be polarized (Qiao *et al.* 1995; Qiao *et al.* 2001). However in cultured isolated ureteric bud, investigations using confocal microscopy do not reveal any difference in the formation of adherens junction proteins (such as ZO-1 and claudin-3) except for Ksp cadherin which is specifically absent in a subset of cells of the ampulla. In addition, the tip regions of the bud are considered the important mediators of branching and nephron induction. As Potter stated,

> *"All tubular divisions and all nephron induction is mediated by the ampulla and neither can occur when ampullary activity is lost"* (Potter 1972).

The rate of proliferation is highest at the terminal ends of the ureteric bud epithelium, suggesting that this is a centre of growth for the ureteric bud (figure 4.1) (Michael *et al.* 2004). Other recent studies have proposed a cell lineage pathway within the ureteric bud (figure 4.2) (Shakya *et al.* 2005a) which may lead to a greater understanding in how cell identities within the bud are regulated. Cell tracing experiments using chimeric ureteric buds indicate that the cells of the tips give rise to cells of the stalk and also proliferate within the tip to give rise to new tip cells (Shakya *et al.* 2005a). The fact that the tips can give rise to stalk cells as well as

possessing self renewing abilities suggests they are multipotent cells. Also the previous chapter has shown that the tip regions of the ureteric bud can undergo a reversible change in cell identity to a stalk cell character (as evidenced by DBA binding and the cessation of branching). The stalk, on the other hand, displays a relatively low level of proliferation and cell tracing experiments suggest that stalk cells can only contribute to newly forming distal stalk regions. However these cell tracing experiments suggest that stalk cells do not contribute to the tips region of the ureteric bud (Shakya *et al.* 2005a).

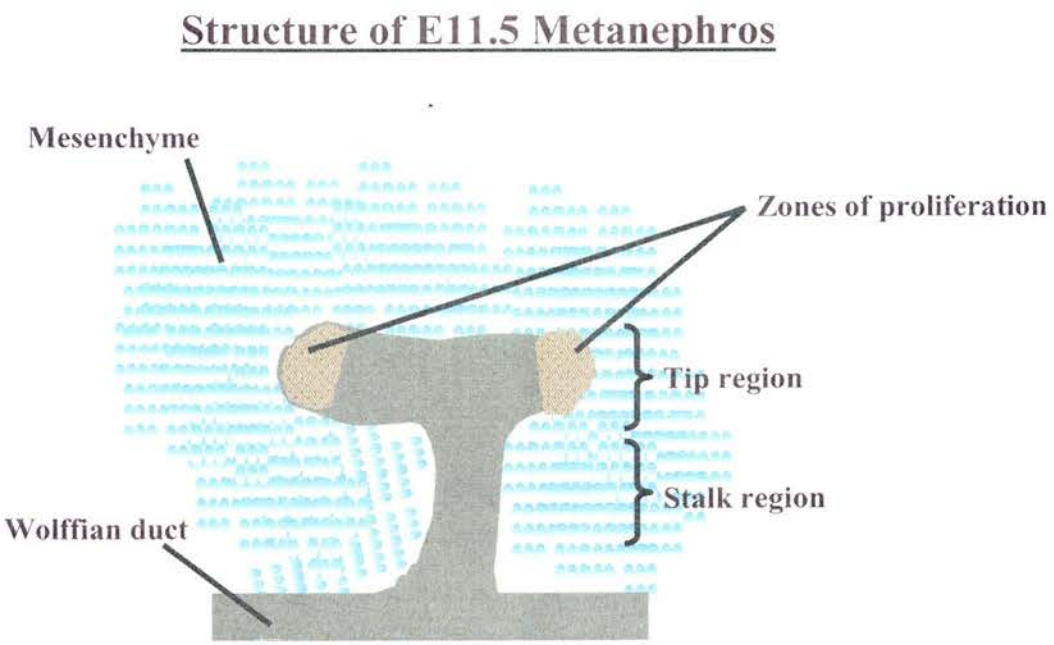


Figure 4.1: Structure of E11.5 metanephros

The zones of highest cell proliferation are located in the ampullae of the tips.

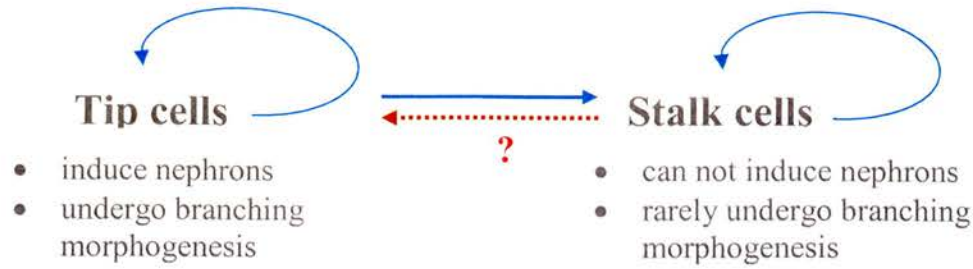


Figure 4.2: Cell differentiation pathway of the ureteric bud.

The tips of the ureteric bud proliferate rapidly and give rise to the cells of the newly forming tip regions and also cells of the stalk region. The cells of the stalk proliferate to a lesser extent and only give rise to additional stalk cells. Stalk cells do not contribute to cells of the tip regions so it unclear whether stalk cells can differentiate into tip cells.

The stalk cells show a lower rate of cell proliferation compared to the tips (Michael *et al.* 2004) but as proliferation still occurs to some extent the stalk regions are not terminally differentiated. Terminally differentiated cells are unable to proliferate (Oshima *et al.* 1991) and are held in this state due to cyclin kinase inhibitors of the cell cycle (Zhu *et al.* 2001). The terminally differentiated cells of the collecting duct system are thought to include principle and intercalated cells (al-Awqati *et al.* 2002; Anglani *et al.* 2004). Cell proliferation in the stalk regions of the ureteric bud is not dependent on GDNF as blocking GDNF signalling in organ culture reduces cell proliferation in the tip regions while the rate of proliferation in the stalks remains unchanged (Michael *et al.* 2004). When branching morphogenesis ceases and the collecting duct system begins to mature, interstitial growth, whereby cell proliferation occurs evenly throughout the ducts, is prominent (Oliver 1968; Potter 1972). The stalk regions exhibit a relatively high degree of cell differentiation as evidenced by the plethora of genes expressed at higher levels in the stalk compared to the tips (Schmidt-Ott *et al.* 2005; Davies 2005). The stalks are in a relatively quiescent state compared to tips (Michael *et al.* 2004). They exhibit a relatively low rate of proliferation, although they are not terminally differentiated. Consequently stalk cells can be thought of as transient amplifying cell population.

It is interesting to note that certain polycystic disease states of the kidney are associated with the collecting duct epithelium (Holthofer *et al.* 1990; Kovacs *et al.*

1997) and are often characterised by increased proliferation and migration of the tubular cells (Ziehr *et al.* 2004; Torres 2004).

The state of differentiation of the stalk is not fully clear but it may be similar to that of other differentiated cells such as the circulating T lymphocytes, which although highly differentiated are not terminally so but are in quiescent growth state (Oshima *et al.* 1991; Brown *et al.* 2003). The T lymphocytes can re-enter the cell cycle however and expand clonally when stimulated by antigen-presenting cells.

It is uncertain whether stalks of the ureteric bud are capable of undergoing branching morphogenesis. Recent *in vitro* studies, which were carried out at the same time as my studies, suggest that it is possible for branches to arise from the stalk region during organ culture with up to 6% of all branching events arising as lateral branches (Watanabe *et al.* 2004). This in itself suggests an intrinsic ability of the stalk regions to form new branches. When the kidneys are cultured in organ culture they flatten down. It is possible that during this period when the kidneys flatten that the tips are restricted somewhat in the rate of growth from the tips and so lateral branches form from the excess production of tip cells. In this way it is unclear if lateral branching is due to the formation of tips cells from stalk cells or whether the lateral branches form due to subsets of tip cells that have been displaced, due to space restrictions, from the tips into the stalk region during the flattening out period of organ culture. In support of this hypothesis, lateral branches have mostly been observed growing from the 2^{nd} generation branching segments (Watanabe *et al.* 2004). These are the branches that must flatten out and adjust to the *ex vivo* culture conditions.

Lateral branching has also been described from 3D culture of isolated ureteric buds but based on the images presented in this paper (figure 4.3) I would describe this branching as terminal trifid branching (Meyer *et al.* 2004). Figure 4.4 illustrates how differential remodelling of the stalk regions can give the false impression of lateral branch formation.

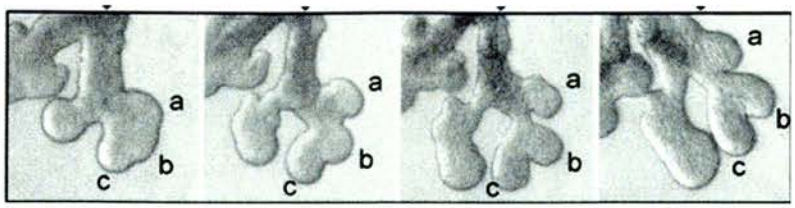


Figure 4.3: Lateral branching described in isolated ureteric bud cultures

This picture is borrowed from Meyer *et al.* 2004. Although the authors describe branch a as a lateral branch with branches c and b resulting from a terminal bifid division, it is also plausible that branches a, b and c form from a single terminal trifurcation division with rapid elongation of the stalk region between branch a and b.

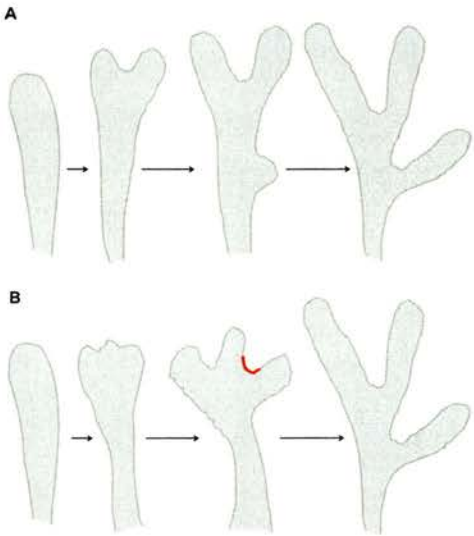


Figure 4.4: Illustration of stalk remodelling to give the appearance of lateral branch formation.

Illustration A shows a single tip branching dichotomously from its terminus with the subsequent formation of a lateral branch from the stalk region. Illustration B shows a single tip branching in a trichotomous fashion from its terminus. Differential remodelling of the stalk region (highlighted in red) can result in the position of the origin of one of the branches lying proximal to the origin of the other two, thereby falsely suggesting that lateral branches form.

In vivo, only terminal divisions (bifid, trifid and carrefour divisions) have been described (Oliver 1968; Potter 1972). As there is no evidence that lateral branches from during *in vivo* metanephric organ development, the lateral branching events that occur *in vitro* may be artefacts of the culture system. Therefore it is unclear whether lateral branch formation is due to stalk cells reverting into tip cells. Overall it is not clear whether stalks can undergo branching morphogenesis. I will present work to investigate this more thoroughly.

The patterning of cells within the ureteric bud epithelium may be a method of restricting branching behaviour to the terminal ends of the epithelium. Branches rarely arise from the stalk regions and this raises two rival hypotheses:

- The stalk regions of the ureteric bud are differentiated to have lost the ability to form branches.

- The stalk regions are suppressed from branching due to repression from the tip regions. In this way the final architecture of the ureteric bud would be explained by adaptive self-organisation of the epithelium itself.

This begs two questions, can stalk regions of the ureteric bud form branches? And if so, can they form branches even when in direct proximity to other tips?

I designed an experiment to discriminate between the two hypothesis using micromanipulation of metanephric kidneys and organ culture.

4.2 Stalk section culture

I tested the hypothesis that the stalk regions of the metanephric kidney do not branch often within the metanephric mesenchyme because they are differentiated and have lost this ability. To test this hypothesis, the tip regions and the Wolffian duct were removed from an E11.5 kidney and the remaining stalk section only was embedded within a mass of metanephric mesenchyme removed from approximately 10 E11.5 kidneys (figure 4.5). These stalk section cultures were maintained for 144hr before being analysed using specific markers for stalk regions and tip regions of the ureteric bud. DBA was chosen as a marker for the stalk region and similarly *Wnt11* was chosen as a marker of tip regions. Both markers have been introduced previously in chapter 3.

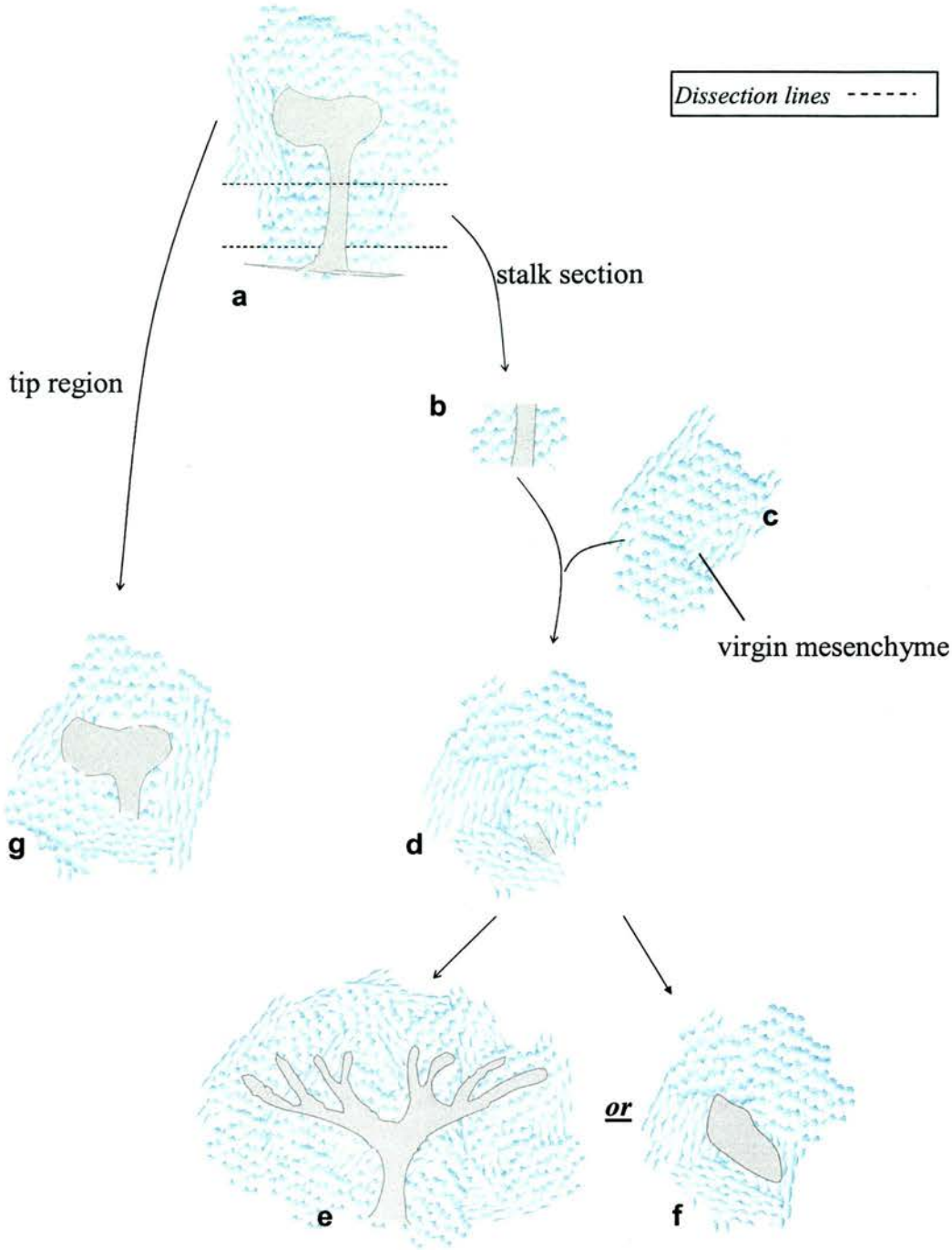


Figure 4.5: Outline of dissection set up for stalk section cultures.

E11.5 kidneys were used to set up stalk section cultures (a). A section of stalk was dissected from the tip region and the Wolffian duct. The stalk (b) was then embedded in extra mesenchyme (c) collected from ~10 kidneys. The stalk section cultures (d) were left to culture for 144hr with medium changes daily, before being investigated to see if they branched (e) or not (f).
The tip regions (g) of the stalk used to set up the stalk section cultures were retained for analysis described in figure 4.6.

As the illustration above suggests, stalks may show the ability to branch when embedded in mesenchyme. However false positive results may arise if, during the set up of the cultures:

- There were tip cells included accidentally with the section of stalk epithelium, or
- there were tip cells included in the mesenchyme in which the section of stalk was embedded.

To guard against these potential errors, control experiments for the dissection procedure were carried out to ensure

- that the section of stalk epithelium did not contain tip cells
- that the mesenchyme in which the section of stalk was embedded did not contain tip cells.

4.2.1 Results

4.2.1.1 Control: Stalk section cultures did not contain epithelium from the tips of the ureteric bud

It was important to dissect sections of stalk from the ureteric bud that did not contain contaminating cells from the tip (figure 4.6). Any included tip cells could themselves generate branches and therefore could give rise to false positive results.

It was impossible to investigate whether the dissected stalk sections were free from tip cells at the start of an experiment without fixing and thereby killing the tissue. The information was therefore obtained from the other kidney tissues.

Based on the chosen markers for tip cells (*Wnt11* expression) and stalk cells (DBA binding), a stalk segment should not express *Wnt11* and should bind DBA throughout its length. As each stalk section was used to set up 'stalk section' cultures (see figure 4.5) for 144hr it was impossible to know if the stalk section was initially *Wnt11* negative and DBA receptor positive. Instead the remaining ureteric bud tissue, containing the tips and a little distal stalk, was analysed to indicate if the stalk section removed from it was beyond the *Wnt11*/DBA boundary and therefore most likely *Wnt11* negative and DBA receptor positive at the time of dissection. The remaining

ureteric bud was therefore cultured for 3hr before being processed for either *Wnt11* insitu hybridisation or DBA lectin fluorescence (figure 4.6). The 3hr culture was needed to allow the tissue to adhere to the filter on which it was cultured so that it would not be lost later during processing. The logic of these controls was as follows:

- It would be highly likely that the removed stalk sections did not include *Wnt11* positive cells if the remaining ureteric bud showed intact *Wnt11* expression domains. 'Intact' means that the *Wnt11* domain did not extend right to the cut site. *Wnt11* in situ hybridisation on the remaining ureteric bud would elucidate this.
- Also, it would be highly likely that the removed stalk sections were DBA receptor positive if the remaining ureteric bud included a section of epithelium that bound DBA i.e. a section of stalk epithelium. DBA lectin fluorescence on the remaining ureteric bud would reveal whether this was so.

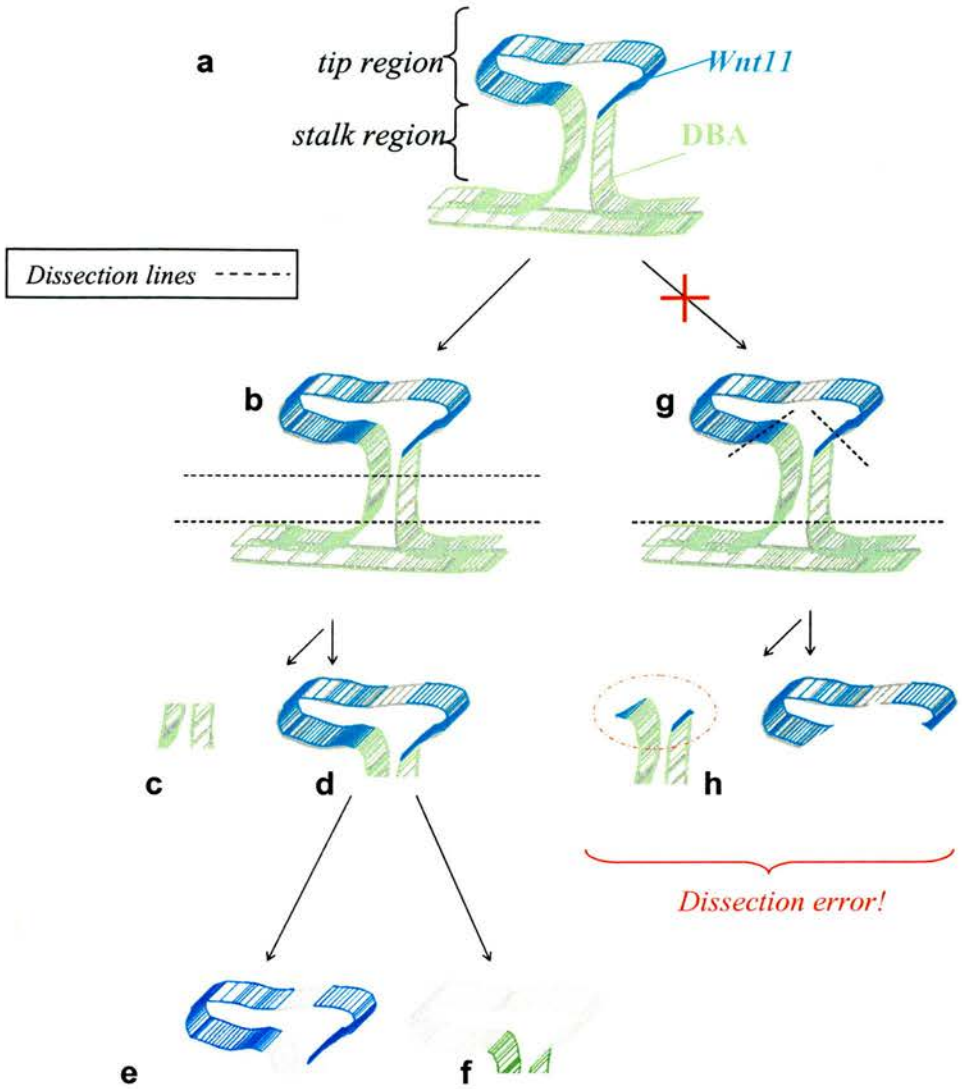


Figure 4.6: Outline of micro-dissections of stalks from E11.5 kidneys

E11.5 ureteric bud is T-shaped with *Wnt11* expression in the tip cells and DBA binding to the stalk cells (a). To remove a section of stalk incisions were made as indicated in b to remove the Wolffian duct and the tip regions. The stalk section (c) was used to set up stalk section cultures (refer to figure 4.5). It would be highly likely that the stalk section (c) would be free from *Wnt11* expressing cells if the remaining ureteric bud (d) had intact *Wnt11* expressing domains. Therefore *Wnt11* expression analysis was carried on the remaining ureteric bud (d) to ensure that the entire *Wnt11* expression domain was left intact (e). It would be likely that the stalk section (c) would express the DBA receptor if the remaining ureteric bud (d) included DBA binding stalk. DBA lectin fluorescence was also performed to ensure that some DBA binding region of the stalk was attached (f).
If, to remove a stalk section, incisions were made as indicated in g there would be a greater chance of contaminating the stalk sections with *Wnt11* positive cells (red circle in h). Any branching morphogenesis seen from these stalk regeneration cultures could due to the contaminating tip cells due to this dissection error. Thereby false positive results could arise.

If the remaining ureteric bud included both the tip and some distal stalk it should contain within it (and go beyond) the entire *Wnt11* domain. When *Wnt11* expression analysis was carried out on the remaining ureteric buds it was revealed that they contained 1 expanded expression domain or 2 separated expression domains of *Wnt11* in the shape of rounded ampullae suggesting that these are the complete tip regions of the ureteric bud (figure 4.7). The single expanded *Wnt11* domain would represent a tip prior to the separation of two individual daughter tips and indicated that there was some slight variation in the stage of branching between dissected kidneys (figure 4.7a). Overall analysis of the remaining ureteric buds for *Wnt11* expression suggest that the tip regions of the ureteric buds have not been dissected away or included with the dissected stalk sections, which were used to set up the stalk section cultures.

Analysis of the remaining ureteric buds using DBA lectin fluorescence, after culture for 3hr, further confirmed that the dissected stalk sections were clear of any contaminating tip cells (figure 4.8). A section of DBA binding epithelium was left included as part of the remaining ureteric bud. This indicates that the incision point to remove stalk section was made within the stalk region itself. Morphologically the tip regions, as seen with anti-calbindin staining, looked full and intact. Therefore the tip cells were not included in the rudiment of stalk section removed to set up the stalk section cultures.

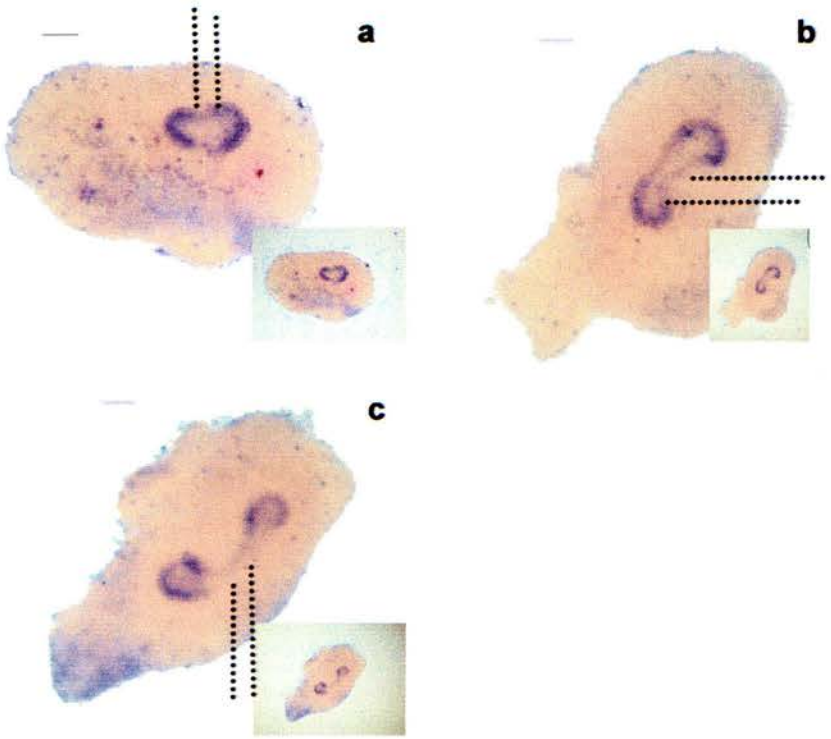


Figure 4.7. *Wnt11* in situ hybridisation of tip regions recovered when stalks were dissected away.

Tip regions were dissected from stalks, cultured for 3hr before being processed for *Wnt11* in situ hybridisation (a, b, c). The intact ampullae of the tip regions were highlighed by *Wnt11* expression. The ampullae maybe be quite close together or more removed from each other (compare a to c) suggesting differences in the degree to which they have undergone the first round of branching morphogenesis. *Wnt11* expression was absent from any remaining ureteric bud epithelium. Therefore it is highly likely that the corresponding stalk regions (implied by the dotted lines) did not express *Wnt11*. These stalk regions would have been used to set up stalk regenerating cultures. The inserts show the images prior to processing using photoshop (the image of the filter on which the kidneys were grown was removed). Scale bar =100μm

.

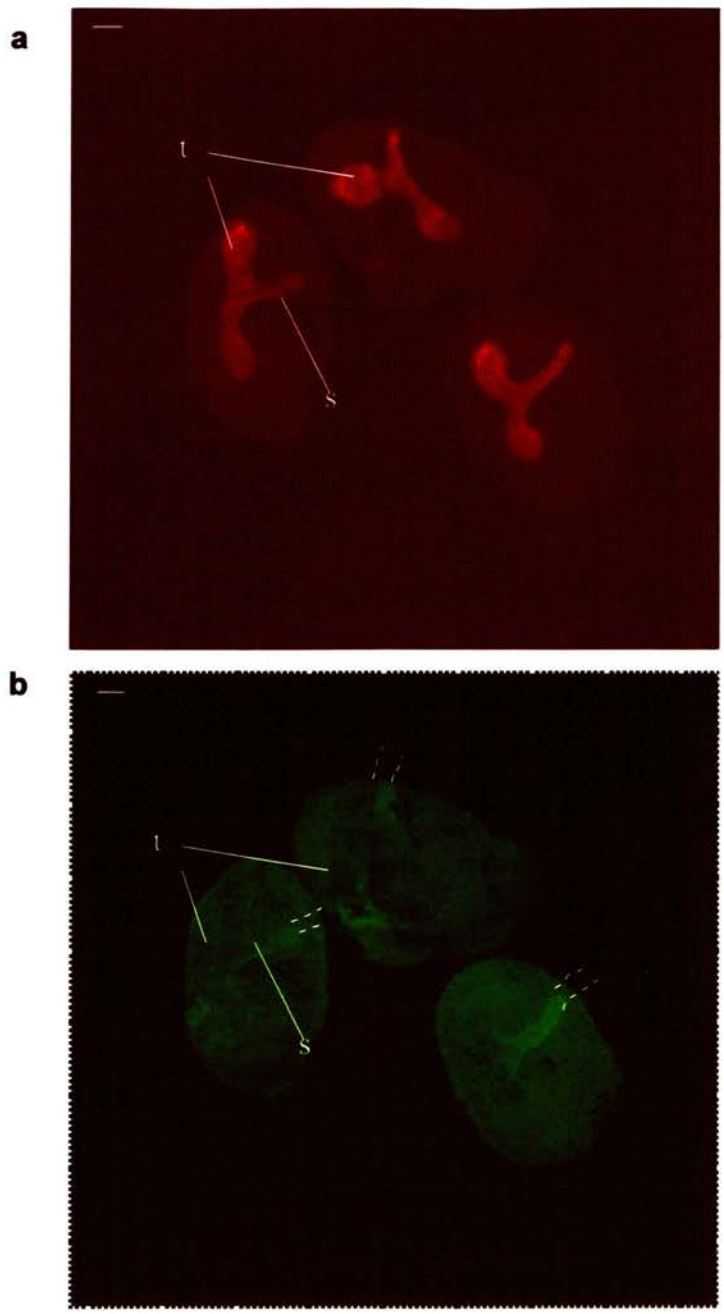


Figure 4.8: Immunofluorescence of tip regions recovered when stalks were dissected away.

Tip regions and stalk regions were dissected from 3 E11.5 kidneys. The tip regions were cultured for 3hr before being fixed. They were stained with TRITC-anti-calbindin-D_{28k} (a) and FITC-DBA (b). The tip regions (t) exhibited intact ampullae as seen in image a. The tip regions did not express DBA except for a length of ureteric bud epithelium close to where the stalk regions (s) were dissected away. This length of epithelium bound DBA and so was the distal portion of the stalk region. The remaining portion of the stalk regions that were removed (represented by the dotted lines in image b) were used to set up 'stalk section' cultures (refer to image 4.9). Scale bar= 100μm.

4.2.1.2 Control: The mesenchyme of the stalk section cultures did not contain epithelium from the tips of the ureteric bud

In order to ensure that the mesenchyme in which the stalk section cultures were embedded did not contain tip cells, mesenchyme was only used if a complete and intact ureteric bud was dissected free from within it. Some stalk section cultures were analysed, after 3hr of culture, for the presence of contaminating epithelium. The stalk section cultures showed a stalk region (which bound DBA over its entire surface) within mesenchyme (figure 4.9). No other epithelium was detected within the mesenchyme (as seen by the anti-calbindin-D_{28k} staining). This confirms that the stalk section cultures only contained a solitary epithelial rudiment taken from the stalk regions of an E11.5 ureteric bud.

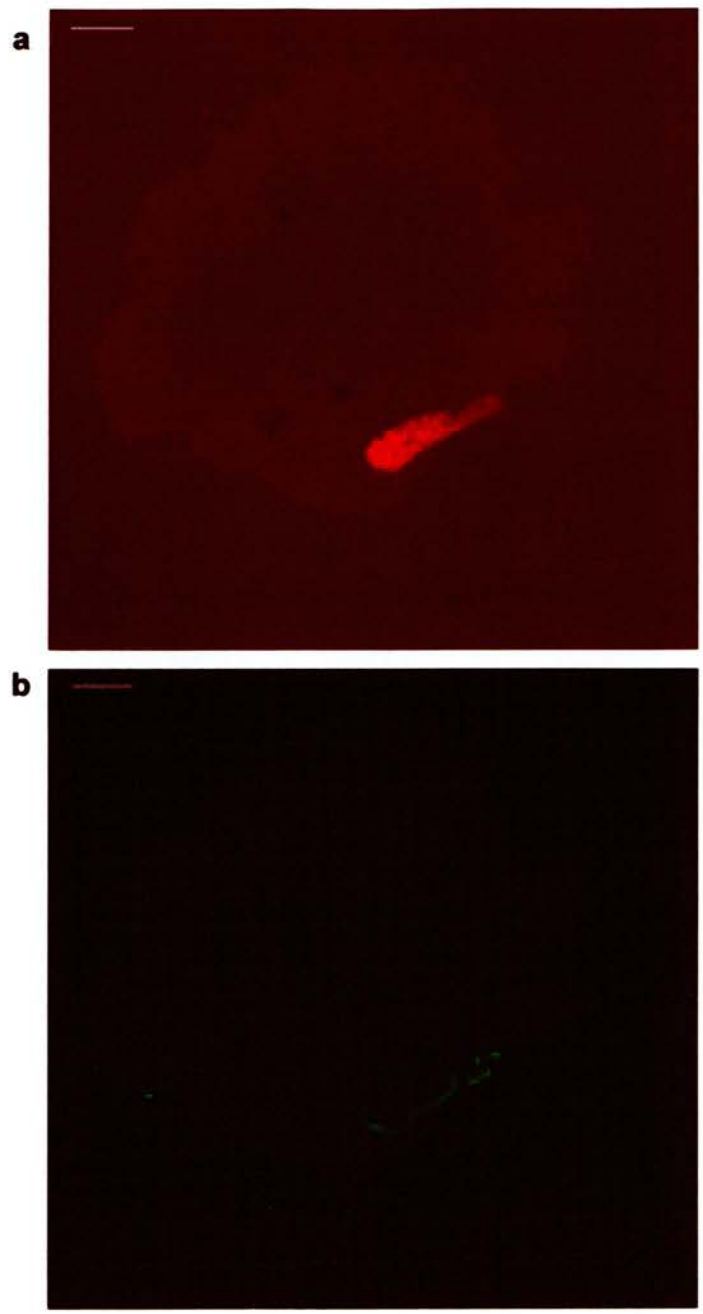


Figure 4.9. Demonstration of stalk regeneration culture set up.

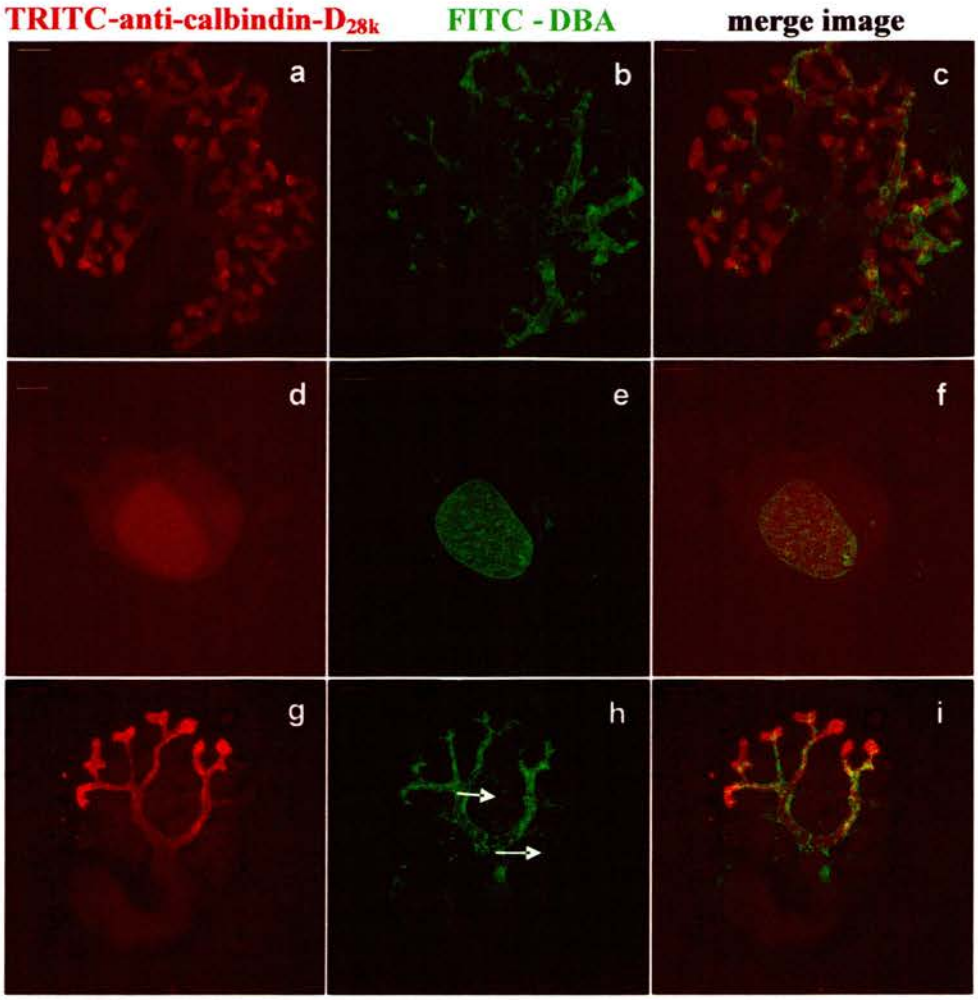
A stalk region was dissected from an E11.5 kidney and embedded within a ball of mesenchyme. This was fixed after 3hr of culture and stained with TRITC-anti-calbindin-D_{28k} (a) and FITC-DBA (b). The stalk embedded within the mesenchyme was expressing calbindin and binding DBA as expected. The surrounding mesenchyme was free from other contaminating ureteric bud epithelia as it is calbindin negative. Scale bar = 100μm.

4.2.1.3 The stalks of an E11.5 ureteric bud do possess the potential to branch

The stalk section cultures were maintained for 144hr. Analysis for DBA binding and anti-calbindin revealed that 71% of the cultures set up (n=14) showed generation of new tip regions (figure 4.10). The stalk regions had progressed from being an unbranched epithelial rudiment that bound DBA along its entirety, to a well branched epithelial tree which showed relatively low binding for DBA at the ends of the branches. Some of the tips of the epithelial tree showed strong binding for DBA suggesting these tips were not actively branching any more (refer to chapter 3). Generation of tips occurred from the original site of dissection used to remove the tip region and the Wolffian duct. A smaller percentage of the cultures (29%) did not show any ability to recapitulate the process of branching morphogenesis. Instead, these cultures formed a closed cyst-like structure which bound DBA over its entire surface. Although there was no staining done to detect developing nephrons specifically, DBA does bind weakly to nephrons (refer to chapter 3 figure 3.7). The stalk section cultures that did not manage to branch instead formed cysts. These cultures did not show any evidence of nephron formation in the surrounding mesenchyme, unlike the branching stalk cultures which did show some evidence of nephron induction.

Similar results were found when stalk section cultures were analyzed for *Wnt11* expression (figure 4.11). Just over half of the number of cultures set up showed expression of *Wnt11* in numerous discrete domains (55%, n=11). 45% of cultures however did not show any *Wnt11* expression. This suggested that the stalk regions of the metanephric kidney can generate tips with proper expression of tip markers such as *Wnt11* and can branch well within the metanephric mesenchyme.

Overall these experiments show that stalk regions of the ureteric bud have some capacity to from functional tip regions.

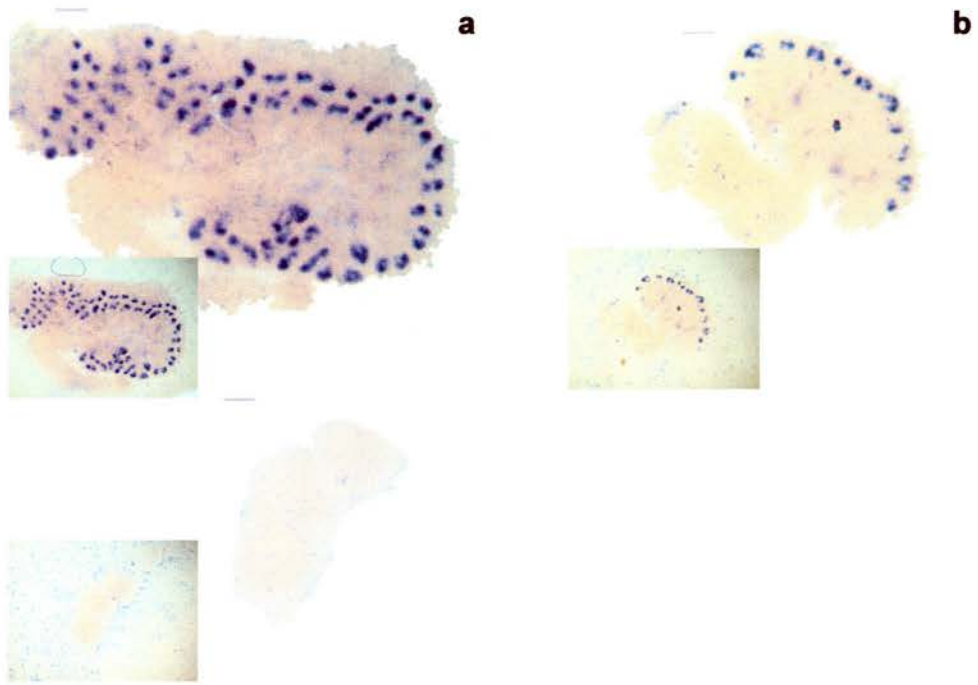


j

No. of stalk section cultures set up	14
% of stalk section cultures showing generation of tips 144hr of culture	71%
% of stalk section cultures not showing generation of tips 144hr of culture	29%

Figure 4.10: DBA and anti-calbindin-D_{28k} staining of stalk section cultures after 144hr of culture.

Stalk section cultures set up as shown in figure 4.5 were cultured for 144hr (d-i). Whole kidneys were cultured as a control (a-c). Cultures were fixed and stained with FITC-DBA and TRITC anti-calbindin-D_{28k}. Merge images are also shown (c, f, i). Whole kidneys branched well and formed numerous tips which did not bind DBA (a-c). 29% of stalk section cultures set up underwent branching and seemed to form a dilated epithelial sphere which bound DBA over its entire surface (g, h, i). 71% of the stalk section cultures set up underwent multiple rounds of branching to form several tips that did not bind DBA (d, e, f,). When tip generation occurred it appeared to be from the site of dissection. Some nephron formation was suggested in the successful generating cultures (arrow). Table j summarizes the results. Scale bar =100μm



d

No. of stalk section cultures set up	11
% of stalk section cultures showing Wnt11 expression after 144hr	55%
% of stalk section cultures not showing Wnt11 expression after 144hr	45%

Figure 4.11: *Wnt11* in situ hybridisation of stalk section cultures after 144hr of culture.

Stalk section cultures set up as shown in figure 4.5 were cultured for 144hr (b, c). Whole kidneys were cultured concomitantly as a control (a). Cultures were fixed and processed for *Wnt11* insitu hybridisation. Whole kidneys displayed numerous *Wnt11* expressing tip domains as expected after 144hr of culture (a). 55% of the stalk section cultures set up showed *Wnt11* expression in the reformed tips as seen in b while 45% of cultures failed to show any *Wnt11* expression (c). Table d summarizes the results. The inserts show the images prior to processing using photoshop (the image of the filter on which the kidneys were grown was removed). Scale bar =100μm

4.3 To investigate if stalks are repressed by attached tips from forming branches, outline of kidney-stalk cultures.

The intrinsic branching processes of the ureteric bud, if they exist, aim to produce optimally spaced out branches. Branch patterns for various kidneys and isolated ureteric bud cultures are similar (Lin *et al.* 2003; Meyer *et al.* 2004). So it is plausible that as part of the intrinsic branching processes of the ureteric bud there are controls on branch formation. The previous experiments tested whether branches grow from the tips because the stalks are incapable of generating tips. However it is now clear that the stalks of the ureteric bud can produce behaviourally and functionally normal tip regions. It is possible that the stalk section cultures from the previous experiment only showed branching behaviour because the tips had been removed. Perhaps the ureteric bud tips themselves, as a control on branch formation (and thereby branch spacing), actively suppress branching from the stalks (figure 4.12). Should a signal to suppress aberrant branch formation emanate directly (through the epithelium) from the tips, the stalks of a kidney would not be able to form branches if tips were attached.

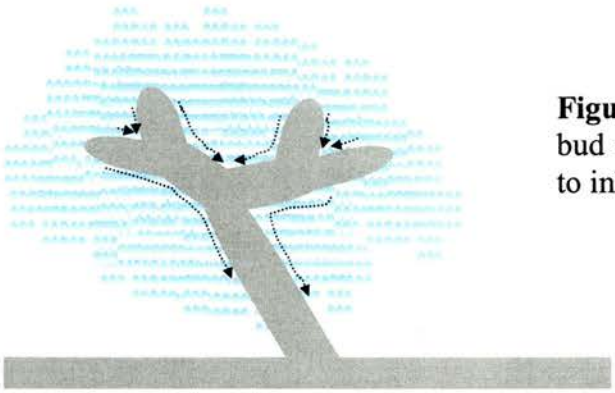


Figure 4.12: The tips of the ureteric bud may signal directly to the stalks to inhibit aberrant branch formation.

The proposed theory suggests that the stalk regions are repressed by the attached tips from forming branches. To test this hypothesis, whole E11.5 kidneys were set up in culture with their stalk regions embedded within extra mesenchyme from approximately 10 kidneys (figure 4.13). Mesenchyme attached originally to the stalk was not removed completely but was trimmed down before the extra mesenchyme

from 10 kidneys was packed around the stalk. These 'kidney-stalk' cultures were maintained for 144hr before being investigated to see if the stalk region branched or not. Analysis was carried out by DBA lectin fluorescence to investigate if branches formed or not from the stalk region of the kidney.

- If branching morphogenesis did occur from the stalk region when tips were still attached, then it is unlikely that the repression signal propagates directly through the epithelium.
- If branching morphogenesis did not occur from the stalk region when tips were attached then it is plausible that tip regions suppressed aberrant branch formation in the stalk via a signal which propagates directly through the epithelium.

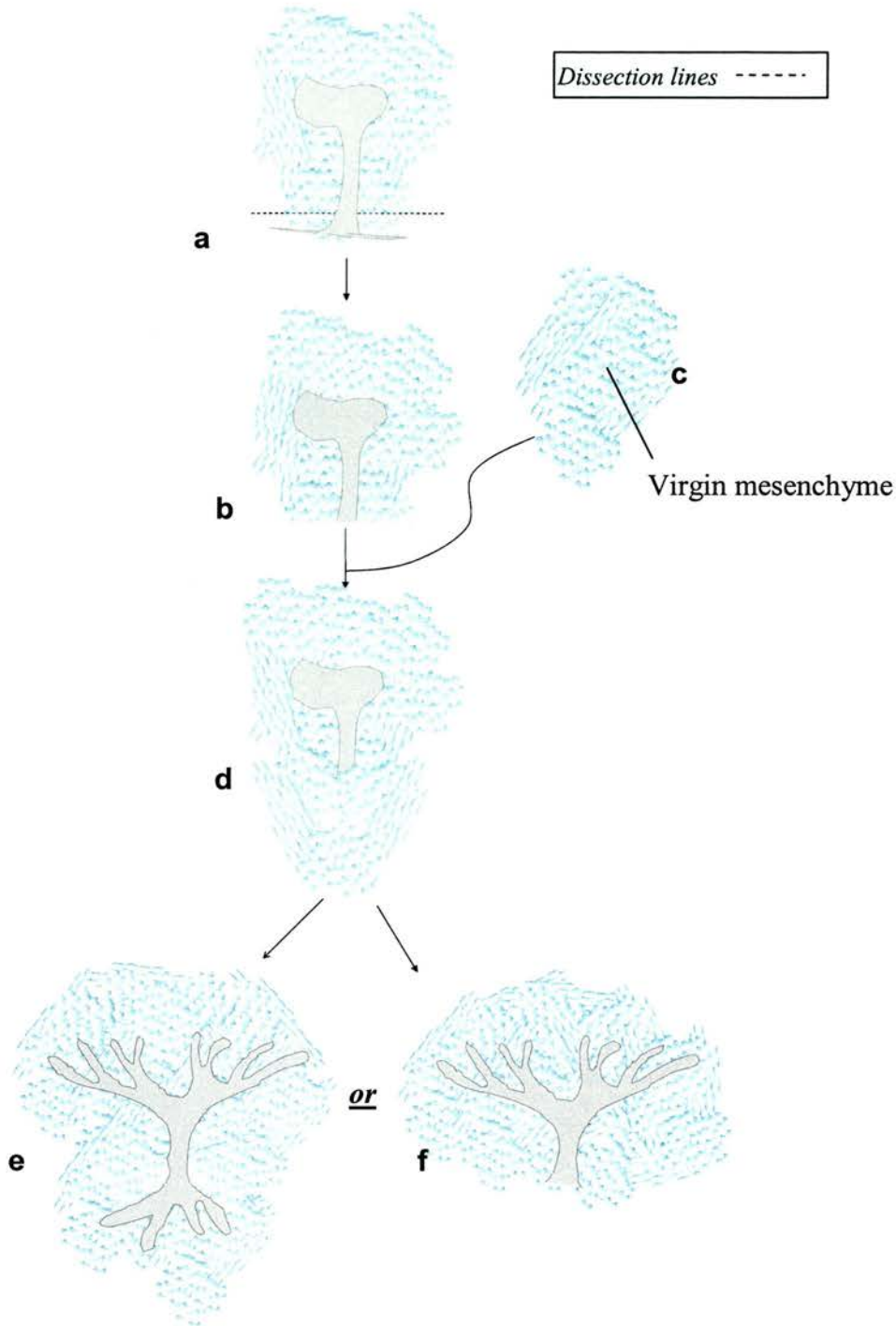


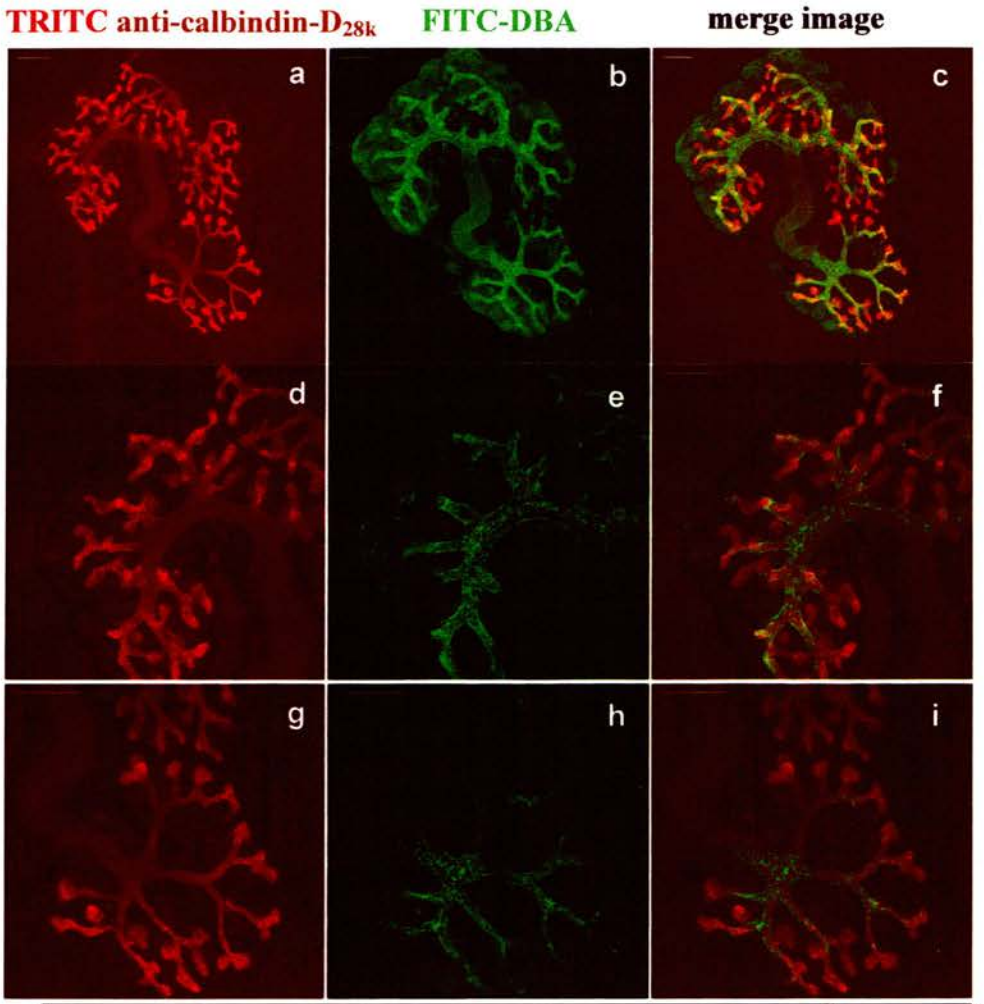
Figure 4.13: Outline of dissection set up for kidney-stalk cultures.

The Wolffian duct was trimmed away from an E11.5 kidney (a). The tip regions were not dissected away. The stalk section of the kidney (b) was then embedded in extra mesenchyme (c). The kidney-stalk cultures (d) were maintained for 144hr with medium changes daily, before being investigated to see if they underwent branching morphogenesis (e) or not (f) from the stalk.

4.3.1 Results

4.3.1.1 The stalk regions of an E11.5 kidney can branch even when there directly attached to tips

Kidney-stalk cultures were set up as outlined in figure 4.13. They were fixed after 144hr of culture before being stained with DBA and anti-calbindin-D_{28k}. 40% of the kidney-stalk cultures set up (n=10) showed branching from the stalk region (figure 4.14). These cultures resulted in the formation of a double headed kidney with two well branched epithelial trees which were joined by a common closed ureter. In such cultures, where the two epithelial trees had undergone multiple rounds of branching, it was difficult to know which tree developed from the stalk region and which tree developed from the tip regions of the original kidney. Both epithelial trees showed a normal (differential) binding pattern for DBA; The tips of each tree showed relatively low affinity for DBA compared to the stalk regions as is seen in a normal kidney. This set of experiments suggest that the stalk region of the ureteric bud can branch even though there are preformed tips elsewhere in the epithelium This suggests that the tips of the metanephric kidney could not be directly responsible for repressing branch formation in the stalks of the ureteric bud.



j

No. of kidney-stalk cultures set up	10
% of kidney-stalk cultures showing regeneration from stalk region after 144hr of culture	40%
% of kidney-stalk cultures not showing regeneration from stalk region after 144hr of culture	60%

Figure 4.14. DBA and anti-calbindin-D_{28k} staining of E11.5 kidney-stalk cultures after 144hr of culture.

E11.5 kidney-stalk cultures set up as outlined in figure 4.13 were cultured for 144hr (a-c). They were fixed and processed for staining with with FITC-DBA (b, e, h) and TRITC-anti-calbindin-D_{28k} (a, d, g). The overlay of the images is presented in c, f and i. A double headed kidney was formed with two well branched epithelial trees connected by a common closed ureter. It is difficult to tell which branched tree arose from the original tip region and which one originated from the stalk of the E11.5 kidney used in the set up. However it is clear from the higher magnification images (d-i) of the two epithelial trees that DBA was down regulated in the tips of each. Table j summarizes the results. Scale bar =100μm

The kidneys used in the above experiments were at the T-bud stage (E11.5) when the cultures were set up. However microarray results published very recently suggested that there is a 5-10 fold increase in expression levels of the tip markers *Wnt11, Ret* and *Ros1* (as normalised to *β-actin* mRNA levels) between primary and secondary branching events which suggests that the ureteric bud undergoes functional segmentation between E11.5 and E12.5 (Schmidt-Ott *et al.* 2005). I tried to address this by setting up kidney-stalk culture using E12.5 kidneys instead of E11.5 kidneys. The set up was the same as outline in figure 4.14 and cultures were maintained for 144hr before staining for DBA and calbindin-D_{28k}. Branching morphogenesis did take place in the E12.5 kidney regenerating cultures. Preliminary investigations suggest that stalks can undergo branching morphogenesis even when tips are attached (figure 4.16) at both E11.5 and E12.5.

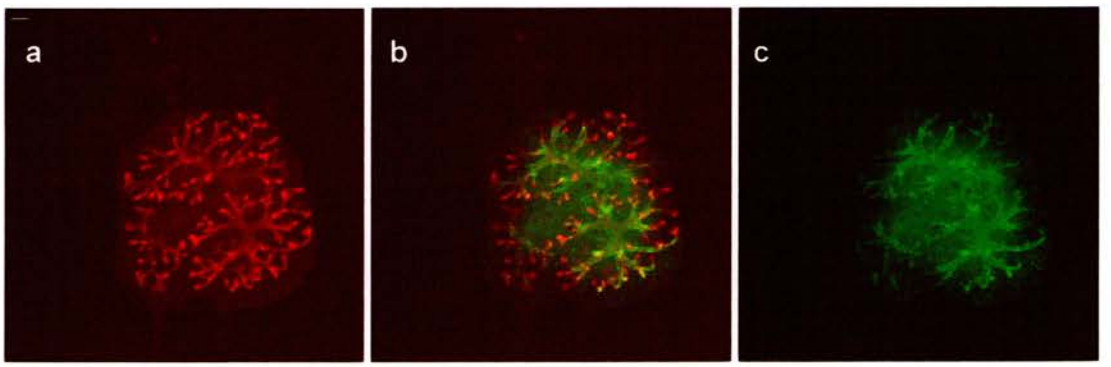


Figure 4.15. DBA and anti-calbindin-D_{28k} staining of E12.5 kidney-stalk cultures after 144hr of culture.

E12.5 kidney-stalk cultures set up as outlined in figure 4.13 were cultured for 144hr (a-c). They were fixed and processed for staining with TRITC-anti-calbindin-D_{28k} (a) and FITC-DBA (b). The overlay of the images is presented in c. Branching morphogenesis occurred from the stalk of the E12.5 kidney. These results are preliminary. Scale bar =100μm

4.4 Summary

The stalks of the ureteric bud are not differentiated to have lost their ability to branch. The stalks can, under certain circumstances, undergo branching morphogenesis either when tips are removed or attached.

4.5 Discussion

The discussion focuses on the following:

- Branch formation from the stalks of the ureteric bud
- The role of metanephric mesenchyme
- Injury repair
- Cell lineage and cell plasticity of the ureteric bud.

4.5.1 Branch formation from the stalks of the ureteric bud

Overall it is evident that the cells of the stalk region are, under certain circumstances, responsive to branching signals. The stalks can generate branches whether or not established tips are attached. This would suggest that the stalks of the ureteric bud are not differentiated to a degree that they have lost the ability to branch and begs further questions as to why, if the stalk cells can branch, don't they do so more vigorously?

Although this work is the first evidence that isolated stalk regions can undergo branching morphogenesis, these findings are in agreement with previous studies carried out on the Wolffian duct. This suggests that the epithelial cells of the ureteric bud are in some ways similar in behaviour from the cells from which they arise, the cells of the Wolffian duct. Supernumerary branching of the Wolffian duct can be induced in culture with GDNF and inhibition of BMP4 (Sainio *et al.* 1997; Miyazaki *et al.* 2000). The Wolffian duct has an added control mechanism which restricts the site ureteric bud formation so that it forms adjacent to the metanephric blastema. This control mechanism involves SLIT2 (secreted protein) mediated ROBO2 (receptor) signalling. Disruption (indicated in *Slit2*$^{-/-}$ or *Robo2*$^{-/-}$ mice) results in the development of numerous ureteric buds from the Wolffian duct as well as the formation of ectopic nephrogenic zones within the medulla of the kidney (Grieshammer *et al.* 2004). The mechanisms by which SLIT and ROBO restrict the site of the ureteric bud are unclear but involve suppressing *Gdnf* in the intermediate

mesoderm anterior to the metanephric mesenchyme (Grieshammer *et al.* 2004). It is unclear if similar mechanisms are involved in suppressing branching from the ureteric bud; both *Slit* and *Robo* genes are expressed in the developing kidney (Piper *et al.* 2000; Stuart *et al.* 2003) so the system may have a role in maintaining *Gdnf* expression at the periphery of the organ so as to reduce ectopic budding from the stalk. However exogenous SLIT2 protein does not affect branching or nephron formation during *ex vivo* culture of kidneys (Piper *et al.* 2002) so its role may be limited.

Some of the new tips generated from the stalk show strong binding with DBA suggesting they are not actively branching. This is clearly seen in some tips of the control kidneys as well (refer to figure 4.10) and so may indicate that after 7 days of culture the inductive potentials of the mesenchyme may be exhausted. Overall the regenerated stalk cultures are under-branched compared to an E11.5 kidney cultured for 144hr. This is unsurprising when it is considered that the intact kidney has preformed tips which can branch immediately in culture and the stalk, I assume, has a delay period until it can generate tips before branching. Yet, the under-branched nature of the stalk-section cultures could be due to increased apoptosis especially in the surrounding mesenchyme. It is often considered that the rescue signals for the mesenchyme only emanate from the tips and not the stalks. Unless the mesenchyme receives signals from the ureteric bud, it undergoes apoptosis by default (Koseki *et al.* 1992). This apoptosis of mesenchyme could be increased in stalk-section cultures as the stalk epithelium is relatively small and possibly unable to secrete the rescue factors at all or in sufficient amounts to maintain the 10 kidney mesenchymes packed around it. My thesis research concerned investigating how branching is activated and it was not within the scope of this project to investigate why branching eventually declines in culture. However it would be of interest to see if there is increase apoptosis in such cultures compared to normal kidneys.

There is a substantial failure rate in which tips are not generated from the stalk region in both the stalk-section cultures and the kidney-stalk cultures that were set up. This rate of failure may reflect the experimental error that may exist due to the complexity of the micromanipulations involved in the culture set ups. However it should be considered that this rate of failure may reflect underlying restrictive

controls on branch formation in the stalk region. The stalk regions used in these experiments often had their own associated mesenchyme attached as this made the tissue bigger and easier to handle. It is altogether possible that this 'stalk mesenchyme' would provide inhibitory signals to the stalk to repress branch formation in 100% of the cultures set up. Indeed in recombination culture where a complete naked ureteric bud is recombined with metanephric mesenchyme successful branching does not always occur unless exogenous factors such as GDNF are added (Lin *et al.* 2003).

It must also be considered that some cultures fail to generate tips because they are not embedded within the mesenchyme in a manner sufficient to induce differential growth. As seen in a small fraction of stalk-section cultures set up, a cyst-like structure can result from the stalk epithelium. Although it is difficult to know how growth is occurring throughout this structure it is probable that the expansion is uniform so a dilatory growth is seen rather than a polarised/restricted area of growth needed to produce bud branches. Of course there is the possibility that the surrounding mesenchymal matrix is too uniform in consistency to adapt and conform to the forces the stalk is imposing on it as it tries to branch out. In branching morphogenesis the surrounding matrix needs to provide resistance in places to the growth of a branch in order to shape it (Fleury *et al.* 2002). In other words there needs to be an anisotropic surface tension (Fleury *et al.* 2002). It is likely that the failure rate with some of the stalk-section cultures is due to insufficient survival and remodelling/re-gelling of the surrounding mesenchyme, which was dissected during the set up.

The tip-specific gene *Wnt11* may have a role in establishing polarity within the ureteric bud epithelium. *Wnt11*$^{-/-}$ mice have renal hypoplasia suggesting an important role for *Wnt11* in branching morphogenesis (Majumdar *et al.* 2003). Specific roles of Wnt proteins in establishing polarity in developing nephrons are coming to light (Plisov *et al.* 2001; Stuart *et al.* 2003) although little work have been done on the role of *Wnt11* in ureteric bud polarity. During neural crest migration *Wnt11* is thought to establish a morphogenetic gradient for the cells to move towards and activation of the planar cell polarity in these cells increases their migratory capacities (De Calisto *et al.* 2005). It is clear based on the experiments presented in this chapter that the

patterning of the ureteric bud does not affect the branching potential of the cells at least at these early stages of kidney development. However it is possible that the stalks of proximal branches are at a more mature state than the more distal branches, especially since there are many differentially expressed genes between tips and stalks (e.g. the molecule to which DBA binds). The most obvious polarisation of the ureteric bud epithelium would be along the proximo-distal axis (figure 4.16). If the ureteric bud was flattened out like a sheet of cells it would have a pattern of cells as seen in figure 4.16 with groups of tip cells (represented by the black circles) being positioned at specific sites within the epithelium, in a fractal pattern. It is possible that the stalks are patterned relative to their distance from the tip regions (conceptualised by the grey 'isolines'). When the kidney-stalk cultures generated new tips from the most proximal branches it is plausible that the generated epithelium recapitulates the proximal–distal pattern of the ureteric epithelium (figure 4.17). If so what would be the state of the interface of the two epithelial trees if it was receiving patterning messages from both 'distal' ends? Would it differentiate more rapidly or perhaps more slowly if the gradient is too 'high'? The identification of markers of proximal and distal stalks would be helpful in understanding more fully the process by which stalks mature.

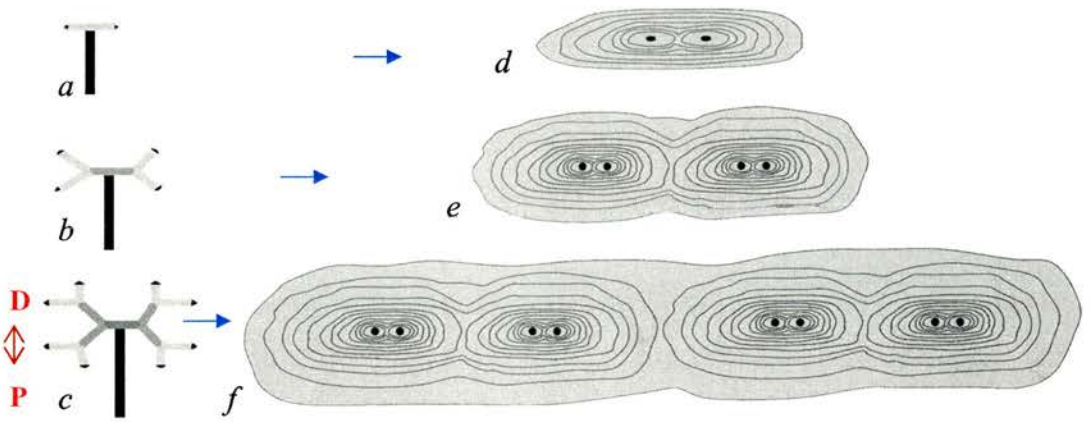


Figure 4.16: The concept of planar cell polarity of the ureteric bud.

Planar cell polarity of the ureteric bud (or any branching epithelium) could be imagined as a patterning of the epithelium in a proximo-distal manner (a, b and c). If the ureteric bud epithelium was laid out as a flat sheet of cells (d, e and f) then the regions of similarly patterned cells (represented by the isolines) would radiate outward from the groups of tip cells (represented by black dots). Distal (D) and Proximal (P).

Figure 4.17: The concept of planar cell polarity of a kidney-stalk culture.

Assuming planar cell polarity is established in the ureteric bud in a proximo-distal manner it is possible that the stalk region regenerates this pattern to form a tip. Once a tip has been generated, the epithelium would have a common stalk region which would be an interface between two 'distally' patterned extremities. Distal (D) and Proximal (P).

4.5.2 The role of mesenchyme

If the controls on branch formation are not due to a determined state of differentiation of the stalk regions then what factors are at play? The surrounding mesenchyme has a potential role in controlling branch formation. Once the ureteric bud has invaded the metanephric mesenchyme, the undifferentiated mesenchymal cells begin to differentiate into at least two cell lineages: nephron cells and stromal cells. The maintenance of a pool of progenitor cells at the periphery of the developing organ means that factors critical for branch formation may be restricted here and the developing branches are induced from the tips of the branches in responses to the morphogenetic gradients (from signals other than GDNF (Shakya *et al.* 2005b)) which are set up at the periphery (Bush *et al.* 2004). The distinction between stalk and tip cells is seen by the array of differentially expressed genes that have been uncovered (Schmidt-Ott *et al.* 2005). Similar microarray prolifing of tip-associated mesenchyme and stalk-associated mesenchyme would elucidate whether there is also differential gene expression in the mesenchyme and possibly highlight genes which are involved in controlling branching morphogenesis.

Particularly good candidates of molecules, which may impose controls on branch formation from the distal regions of the ureteric bud, are BMP4 and sonic hedgehog (figure 4.18). BMP4 is expressed by the mesenchyme surrounding the stalks and Wolffian duct and has been shown in culture to inhibit branch formation and promote elongation of the ureteric bud (Miyazaki *et al.* 2000). *Wnt11* expression is also reduced by BMP4 (Miyazaki *et al.* 2000) suggesting it can convert tip cells to the stalk phenotype. BMP4 expands the population of periureteric smooth muscles cells around the stalks (Raatikainen-Ahokas *et al.* 2000). Interestingly sonic hedgehog, expressed by the stalk cells themselves, is thought to maintain this pathway by up-regulating *Bmp4* expression in the adjacent mesenchyme (Yu *et al.* 2002). However removal of sonic hedgehog signalling from the kidney results in hydroureter and hypoplastic kidneys and no abberant branch formation was described so it is possible that sonic hedgehog only has a limited role and is only important for patterning more mature stalks.

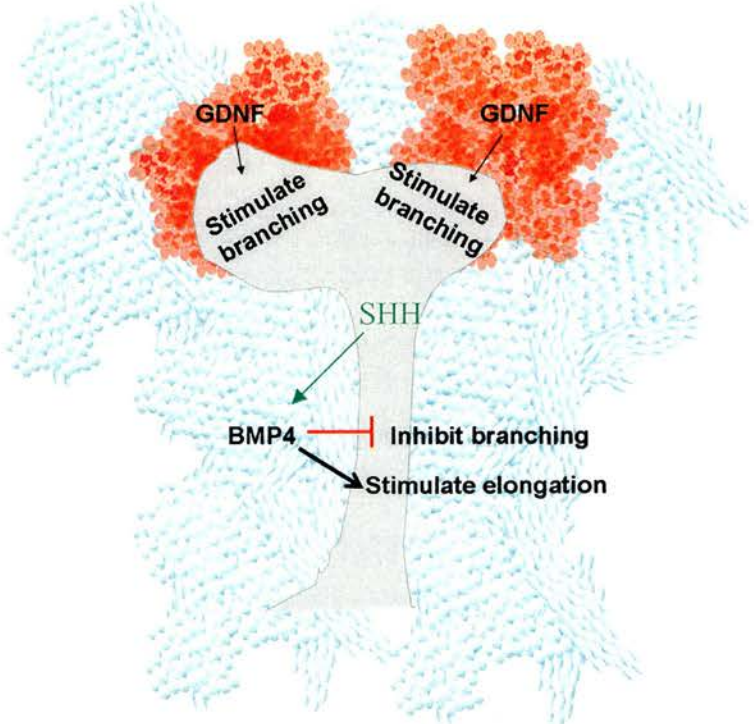


Figure 4.18: Regulation of branching morphogenesis by BMP4.

BMP4 is expressed by the mesenchyme and is thought to inhibit branching in the distal stalks while stimulating elongation. Sonic hedgehog is known to induce *Bmp4* expression and *Shh* is expressed by the cells of the proximal collecting duct from E14.5. This illustration is based on the illustrations in Miyazaki *et al.* 2000 and Yu *et al.* 2002.

Although cell culture models of branching morphogenesis suggest that branching behaviour is intrinsic to the epithelial cells themselves, these models do not recapitulate the branching processes perfectly. With mIMCD$_3$ cells, and possibly other cell types which undergo branching in gel culture, the branches that form are spicular in shape and appear to arise randomly off the original cyst structure into the surrounding artificial matrix. They undergo branching by extending filopodia, unlike the budding process seen with the ureteric bud (Meyer *et al.* 2004). Isolated culture of the ureteric bud however shows a pattern of branching which is more similar to that of the ureteric bud in mesenchyme (Meyer *et al.* 2004) and the authors also note the initiation of branches from the stalks of the ureteric buds in this culture system which lends support for the role of the mesenchyme in repressing branching morphogenesis from the stalk (Meyer *et al.* 2004). Meyer *et al.* also recognise the 'quality control' role of the mesenchyme in shaping and restricting branches during kidney organogenesis. Mesenchyme is composed not only of cells but also of matrix which may provide a precise structure for guiding branches. The importance of mesenchyme controlling branching was demonstrated through experiments in which ureteric buds were grown in heterologous lung mesenchyme (Lin *et al.* 2001; Vainio *et al.* 2003). In these experiments the ureteric bud was maintained and grew well within this mesenchyme but produced a monopodial branching pattern similar to that seen with lung organogenesis. As a result, it is thought that the mesenchyme provides the motogenic and mitogenic signals in a restrictive fashion and also provides a suitable scaffold to shape and remodel the branches of the epithelium as they grow. It is plausible as well that the tips suppress branch formation in the stalks by indirect signalling outside of the epithelium, possibly via secreted factors which act as branch suppressors. Such a factor may effect the mesenchyme itself but as the ureteric bud branches in a controlled manner in a 3D matrix (Meyer *et al.* 2004) it is possible that this factor is an extracellular signal which is left behind in the matrix/mesenchyme as the tips pass through it.

The patterning of a branching epithelium into branching and non-branching regions is not only obvious in the developing kidneys but can be seen in other

branching organs such as the lung. The design of the embryonic lung has similarities with the embryonic kidney as it is considered to contain two regions, a proximal non-branching region corresponding to the main bronchi and the lower third of the trachea, and a branching region that lies distal to the bronchi. Microarray studies have highlighted the differential gene expression between branching and non-branching regions of the mouse embryonic lung suggesting a patterning of the branching of the lung epithelium in a proximo-distal fashion as seen with the kidney (Lu *et al.* 2004). Micro dissection experiments explored the branching potential of tracheal (typically non-branching) epithelium within mesenchyme from the tracheal and distal lung regions of the developing lung (Shannon *et al.* 1998). Findings conclude that the tracheal epithelium can branch similarly to the distal lung epithelium and in fact can undergo cytodifferentiation to the distal cell types. Importantly cell identity and branching behaviour of the lung epithelium is specified by the mesenchyme, with the tracheal mesenchyme suppressing branching in both the trachea and distal lung epithelium while the distal lung mesenchyme encourages branching of both epithelial segments (Shannon *et al.* 1998). Cyst-like structures formed when the trachea or distal lung epithelium was embedded in tracheal mesenchyme. These encysted stalks may be comparable to the cysts seen when the stalks of the ureteric bud failed to generate branching tips (Shannon *et al.* 1998). Overall these investigations highlighted the important role the mesenchyme carries out in regulating branching morphogenesis in the lung. It is possible that similar processes may be occurring during kidney development with the mesenchyme of the stalk discouraging branch formation and the tip mesenchyme encouraging it.

4.5.3 Injury repair

Although without time lapse imaging it can not be assumed for certain, in both the stalk-section cultures and the kidney-stalk cultures generation of tips from the stalks always occurred at the site of dissection. Parallels have long been made between the molecular processes involved during development and those involved in repair of adult tissues. In the kidney it is also thought that the molecular pathways of renal repair recapitulate those of kidney organogenesis (Sakurai *et al.* 1998; Abbate

et al. 1999; Bonventre 2003; Devarajan *et al.* 2003) and in fact developmentally important signalling molecules such as TGFβ, BMP4 and HGF show therapeutic potential in aiding renal repair (Liu 2002; Zeisberg *et al.* 2003). Therefore it should be considered that the positive tip regenerative abilities of the stalks in these experiments could be due, at least in part, to the reparative processes of the epithelium. It is altogether plausible that the tips are generated because the stalk epithelium reverts to a dedifferentiated state in response to the incision injury. One of the most obvious responses of any epithelium when damaged is to increase local cell proliferation and migration in order to close the edges of the wound (Beuerman *et al.* 1992; Moll *et al.* 1998). Rho GTPase activation also occurs during wound healing and is necessary for cell migration and injury repair (Anderson *et al.* 2000; Nobes 2000; Fukata *et al.* 2003; Desai *et al.* 2004). Rho activation also mediates branching morphogenesis of the ureteric bud (Michael *et al.* 2005). If such processes occur in the regenerating cultures it is possible that the localized cell proliferation becomes the basis of and maintained, possibly by signals from the surrounding mesenchyme, as a new zone of growth. It may in fact be the case that branching behaviour can only occur at areas where epithelial continuity has been weakened or compromised. In accordance with this hypothesis it appears that the ureteric bud tips themselves display a discontinuous basal lamina (Qiao *et al.* 1995). Meyer *et al.* claim that in an isolated ureteric bud culture the basal lamina remains continuous throughout but in the electron micrographs presented in this paper I can see little evidence of a basal lamina so it's difficult to tell if it is discontinuous. In my opinion these images show that the basal cell surfaces of the ureteric bud are continuous with each other and show no disruption by cytoplasmic extension. Precision experiments involving injury of the epithelium would help answer this question although laser dissection would have to be employed. I attempted to carry out experiments based on this hypothesis using needle dissection methods but it was difficult to injure the stalk epithelium at precise sites and in a repeatable manner without transecting the epithelial tube completely.

To highlight further the importance of localised degradation of components of the basement membranes in regulating branching it is interesting to note that cleavage product of the basement membrane component collagen XVIII, endostatin,

is found associated with branch points and is a negative regulator of branch formation (Karihaloo *et al.* 2001). *Collagen XVIII* is expressed by the stalks of the ureteric bud and the tips of the developing lung epithelium (Karihaloo *et al.* 2001; Lin *et al.* 2001). Matrix metalloproteinases originating from the tips of the ureteric bud are thought to convert the collagen XVIII into endostatin thereby inhibiting branch formation in the stalks (Barasch *et al.* 1999; Pohl *et al.* 2000).

4.5.4 Cell lineage and cell plasticity of the ureteric bud

The generation of tip regions from the stalks of the ureteric bud provides evidence to suggest that the stalks of the ureteric bud are not differentiated to the extent that they to have lost their ability to form branches. Therefore the production of stalk cells from tip cells does not correspond to an irreversible differentiation pathway. It is more plausible that stalk and tips cells represent stable interchangeable states of the same cell type, the epithelial cell of the ureteric bud (al-Awqati *et al.* 2000). It is altogether possible that the two alternative states of a single type may be a 'rapidly dividing' state and a 'quiescent' state as seen with tip cells and stalk cells. In this sense the stalk cells are specified, but not yet determined to differentiate into the various terminally differentiated cell types of the collecting duct system (al-Awqati *et al.* 2002) (figure 4.19).

Differentiation is the functional specialisation of cells and is often recognised as a change in the profile of expressed genes. Differentiation is often simultaneous with but independent of cell proliferation (Brown *et al.* 2003). A special case of differentiation is terminal differentiation in which cells do not proliferate (and cell senescence can also be thought of as terminal differentiation (Oshima *et al.* 1991)). Non-terminally differentiated cells can switch between actively proliferative and quiescent states (Oshima *et al.* 1991). Maturing cells initially become specified which means they begin the process of differentiation but can reversibly differentiate to revert to the initial cell state (al-Awqati *et al.* 2004). Once a cell is determined however it can not reversibly differentiate and is committed to a certain cell fate (al-Awqati *et al.* 2004). Whether the new cell fate to which it commits itself is terminal

differentiation or just another level of differentiation depends on the stage of maturation.

There is little evidence that primary cells of the adult collecting duct can undergo branching morphogenesis apart from $mIMCD_3$ cells which are a transformed cell line (Rauchman *et al.* 1993). Primary cells derived from pieces of P10-14 mouse kidney formed branched tubules when embedded in matrigel matrix and the structures were thought to be collecting duct cells because the cells had centrally located nuclei with distinct cell borders and processed microvilli (Taub *et al.* 1990). However molecular characterisation of these branching primary cells was not carried out to confirm a collecting duct phenotype and the authors recognise the possibility that the branching cells could be mesenchymal in origin (Taub *et al.* 1990). Another report suggests that primary cells derived from adult renal tissue can self assemble into glomerular and tubular structures although again it is unclear from this abstract report whether these tubules refer strictly to collecting duct cells, nephron cells or both (Joraku *et al.* 2005).

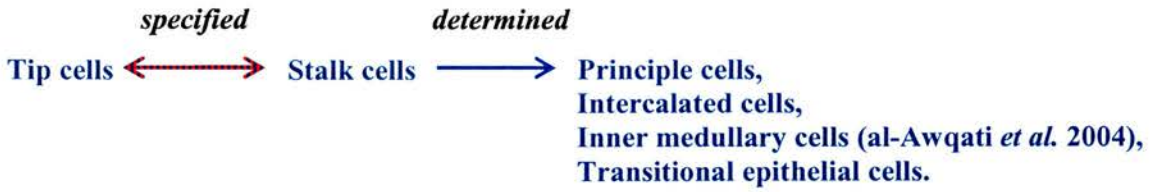


Figure 4.19: Proposed differentiation pathway of cells of the ureteric bud.

Tip cells can give rise to stalk cells and tip cells. Stalk cells can give rise to more stalk cells and under certain circumstances can also give rise to tip cells. Perhaps it is when stalk cells become determined that they appear to loose their ability to form tip cells and differentiate into various cell types of the collecting duct system.

The stalk-section cultures suggest that at least until E12.5 the cells of the stalk are not yet determined to become terminally differentiated cells. This raises the question, when do the stalk cells commit to a terminal cell fate? Cell cultures derived from the E15.5 the ureteric bud behave morphologically like principle cells in that they possess a single apical cilium, but no intercalated cells are seen in these cultures

(Huber *et al.* 1996; Huber *et al.* 1999). These cells using patch clamp analysis show whole cell currents similar to those of a freshly dissected ureteric bud (Huber *et al.* 1999). However these cells represent an immature principle cell compared to those of the mature cortical collecting duct which show different whole cell currents (Huber *et al.* 1996). These experiments suggest that the ureteric bud cells undergo an apico-basolateral polarisation closer to/after birth (Huber *et al.* 1996) although the genes necessary to establish a mature cell phenotype are beginning to be expressed as early as E15.5. Studies in human foetal kidneys at 12 weeks show that the ureteric bud cells express the principle cell marker aquaporin2 with no detectable expression of intercalated cell markers (H^+-ATPase) at this stage. Unfortunately there is no distinction made between the tip and stalk regions of the ureteric bud (Devuyst *et al.* 1996) in these studies. In the rat, aquaporin2 is expressed at low levels at birth and levels increase in the neonatal period (Liu *et al.* 2005). Expression studies for markers of principle and intercalated cells have not yet been analysed during early stages of kidney development (as early as E11.5) but it would be interesting to see if their expression coincides with the appearance of stalk cell markers. Also of interest would be to investigate when during collecting ducts maturation the potential to branch is lost, if in fact it ever is.

It is apparent that the adult cells derived from the stalk regions show some plasticity of behaviour themselves. The β-intercalated cell of the collecting duct is suggested to be the stem cell precursor for both the α-intercalated cell and the principle cell (Fejes-Toth *et al.* 1993) and these cell changes are regulated by the electrolyte environment (Steiner *et al.* 1997). So it may be the case that stalk regions represent immature principle-like cells that convert during maturation into mature principle cells and β-intercalated cell (which themselves show multiplasticity to convert into α intercalated cells and principle cells) (figure 4.20). In this way the β-intercalated cells could be thought of as the resident stem cells of the adult collecting duct epithelium.

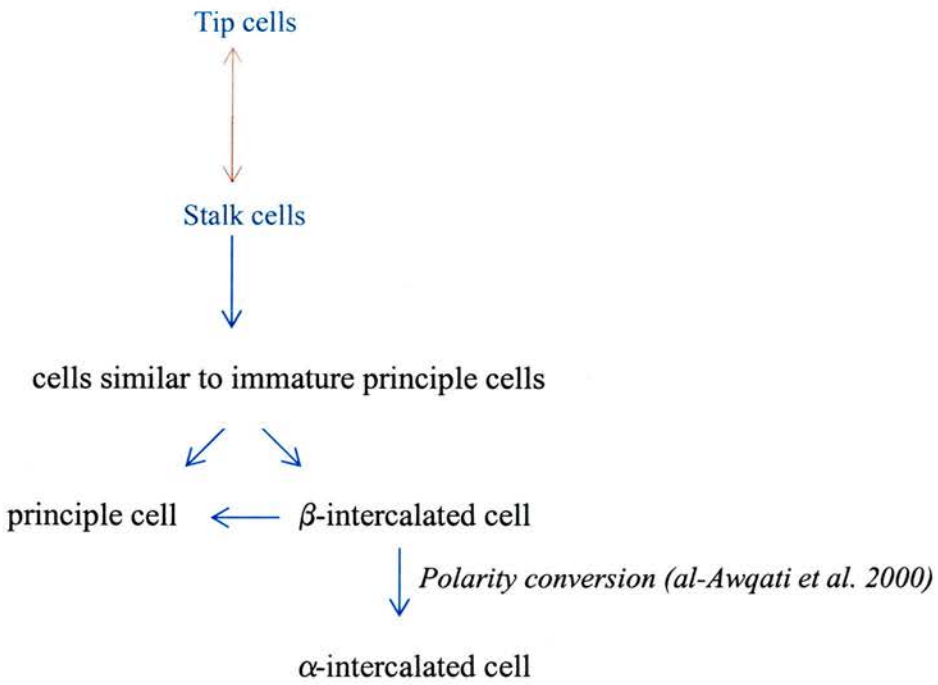


Figure 4.20: Differentiation pathway of cells of the ureteric bud to cells of the adult collecting duct.
It seems that the stalk cells represent an immature from of the principle cells which can develop into principle cells and β-intercalated cell types. The β-intercalated cells can give rise to principle cells and α-intercalated cells.

Potter suggests that branching and nephron induction is only initiated by the ampullae (tips) of the ureteric bud (Potter 1972). I have come to agree with this point of view. The branching morphogenesis and nephron induction that takes place when stalk regions are cultured in mesenchyme can only occur once tips are generated. Without tip regions the stalks of the ureteric bud do not branch or induce nephrons, as neither of these processes was detected in the stalk cultures when the epithelium failed to generate tips and merely encysted. Perhaps the stalks have roles in later stages of nephron development but they do not appear to possess inductive capabilities.

In summary the stalks of the ureteric bud display, under certain circumstances, the ability to branch. They are not differentiated from tip regions to have lost this ability. Other control mechanisms, such as inhibition from stalk

specific mesenchyme or repulsive mechanisms between tips (to be investigated in chapter 5), must be at play during metanephric kidney development to ensure that the pattern of ureteric bud branches forms the optimum epithelial tree.

Chapter 5

Colliding kidneys stop branching earlier than expected and are compressed in the direction of the collision.

5.1 Introduction

Earlier experiments sought to evaluate how the cells of the ureteric bud regulate their identity and branching behaviour. As suggested by the studies in chapter 4, it does not seem the case that long range repressive signals through the epithelium inhibit branch formation from the stalk regions of the ureteric bud. Perhaps the pattern of branching of the ureteric bud is due to other control mechanism of the epithelium. I investigated whether the tips of the ureteric bud have influence on the spatial patterning of their neighbouring branches.

The tips of the ureteric bud are the areas from which inductive signals to the surrounding mesenchyme emanate. The ureteric bud branches may space out in this manner in order that efficient induction of the surrounding mesenchyme is carried out. The tips do not seem to collide with each other but it is unclear how the tips manage to maintain optimal positioning towards the periphery of the metanephros and avoid bumping into each other. The ureteric bud is thought to have its own intrinsic branching program as isolated buds can branch well in mesenchyme free culture and the tips and stalk of these buds show differential gene expression (Meyer *et al.* 2004). Therefore it is possible that within this intrinsic branching program there are controls to achieve adequate spacing of the epithelial tree. Maybe signalling takes place in a repulsive manner between tips of the ureteric epithelium to ensure branches do not collide and that they space out sufficiently. These could be described as tip-tip repulsive interactions (figure 5.1).

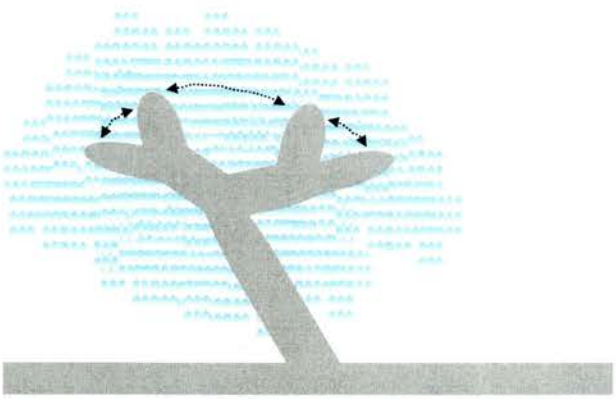


Figure 5.1: Tip to tip signalling. The tips of the ureteric bud may signal to each other so that they do not come too close together, thereby ensuring the correct archetypal pattern of branches is formed.

To investigate whether there are tip-tip interactions taking place during ureteric bud branching morphogenesis, E11.5 kidneys were set up on a collision course with each other to test how they behave when branches are forced to come close together.

5.2 Experimental design

To investigate the effects tips may have on surrounding tips, kidneys were set up in a clustered arrangement (figure 5.2) and maintained for 120hr and 164hr. In this way tips were placed on collision courses with those of neighbouring kidneys and were given the potential to grow in closer proximity than usual. As controls, kidneys were also set up in organ culture in isolation from other kidneys. If tip-tip interactions were involved in controlling branch spacing and arrangement of the ureteric bud it was proposed that the kidneys in clusters could react in a number of ways:

- If the decision of a tip to branch is set by the rule 'do not branch if you are too close to another tip', then the kidneys in cluster may be under branched relative to controls. To test this hypothesis, the average number of tips per kidney was compared between kidneys in cluster and kidneys in isolation.

- If the decision of a tip to grow is set by the rule 'navigate away if you are too close to another tip', then the kidneys in cluster may have an altered branching pattern compared to controls. To test this hypothesis, the eccentricity of the branching pattern produced by each kidney was measured and the average eccentricity was compared between kidneys in cluster and kidneys in isolation.

The spacing of tips of kidneys was examined in two ways. Firstly, I aimed to test whether spacing of the peripheral tips of clustered kidneys was random or not. The relative position of peripheral tips with respect to opposing tips was analysed. Secondly, the idea that the tips of a kidney navigate so that they do not grow within a

certain distance to another tip was examined. To determine if a minimum distance between neighbouring tips is respected the minimum distance between tips was measured and compared between kidneys in cluster and those in isolation.

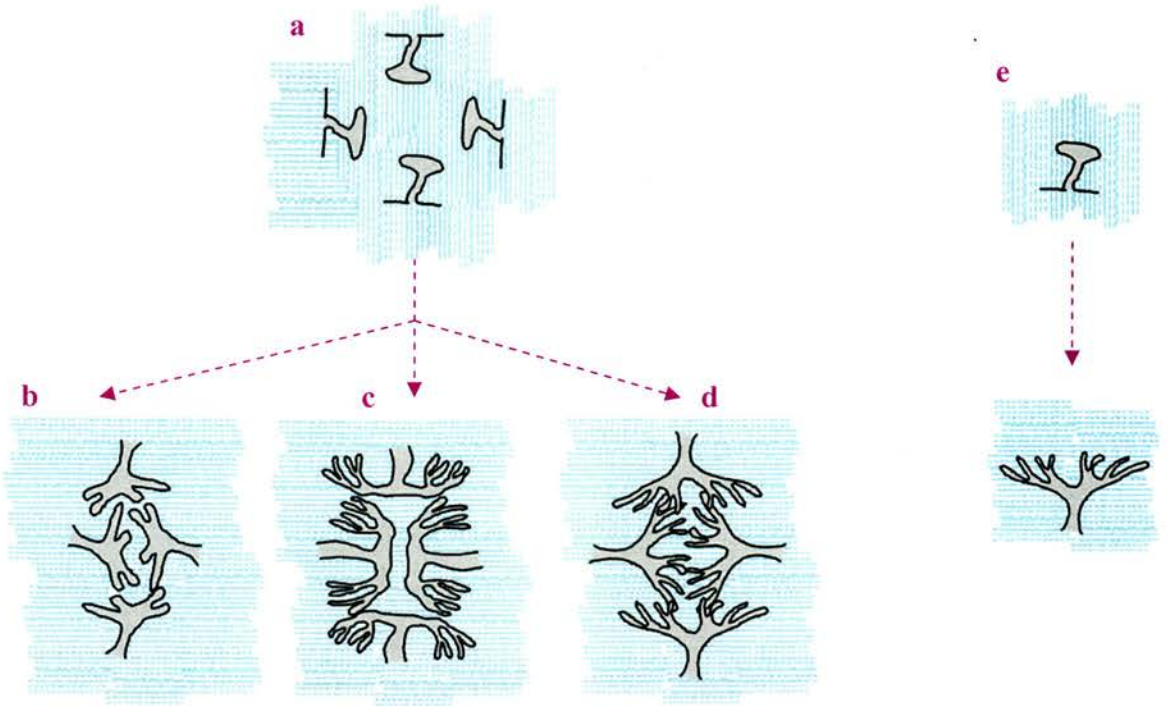


Figure 5.2: Schematic for the kidney cluster experiments.

E11.5 kidneys were dissected and set up in organ culture. In order to force the tips of kidneys onto a collision course with each other, a set of 4 kidneys was set up in a head to head fashion (a). Isolated kidneys were cultured alongside as comparative controls (e). Cultures were maintained for 120hr or 168hr.
There were three possible effects on branching morphogenesis considered when kidneys were set up in clusters. The kidneys may grow but at a slower rate and therefore appear under branched relative to the controls (b). The kidneys may not appear under branched but the spacing of branches may be affected; possibly growing away from each other (c). The kidneys may not show any difference in the number of branches they produce or the arrangement of the branches (d).

5.3 Results

5.3.1 Culture of kidneys on a collision course

Kidneys were clustered in groups of four in a head to head fashion as seen after 24hr of culture (figure 5.3). Cultures were maintained for 120hr (figure 5.4a) or 168hr (figure 5.4b). The images were stained with anti-calbindin-D_{28k} which highlights the ureteric bud epithelium and does not stain the surrounding mesenchymal tissue. The images were converted to greyscale images using Adobe Photoshop and the colour levels of each were adjusted manually to highlight individual ureteric buds from each kidney. The branching patterns were particularly difficult to trace especially in the 164hr cultures where the maturing stalk regions showed weaker staining for calbindin-D_{28k} (figure 5.6). Anti-calbindin-D_{28k} staining also became obvious in the developing connecting ducts (figure 5.5). The 120hr and 164hr cultures (isolated kidneys and kidneys in clusters) did not always show centrifugally-orientated tips and often the tips seemed to face back towards the core of the kidneys instead of facing outwards (figure 5.6).

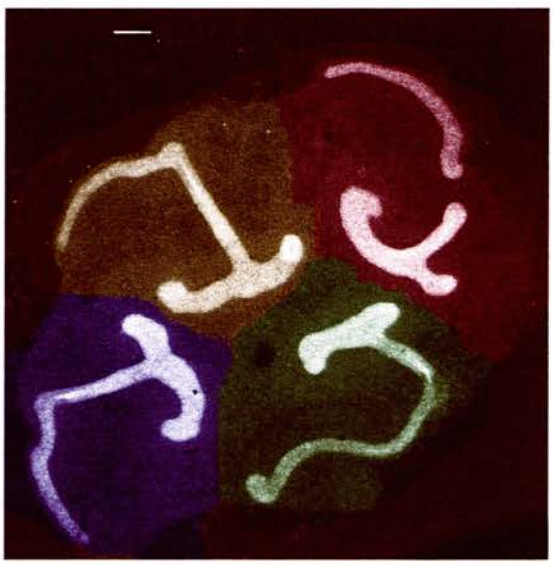


Figure 5.3: Anti-calbindin-D_{28k} staining of a cluster of E11.5 kidneys grown for 24hr.

Kidneys were set up in a head to head configuration and grown for 24hr. They were stained with anti-calbindin-D_{28k}. The image was converted to greyscale and was artificially coloured using Adobe Photoshop to distinguish between the 4 epithelial trees. Scale bar = 100μm

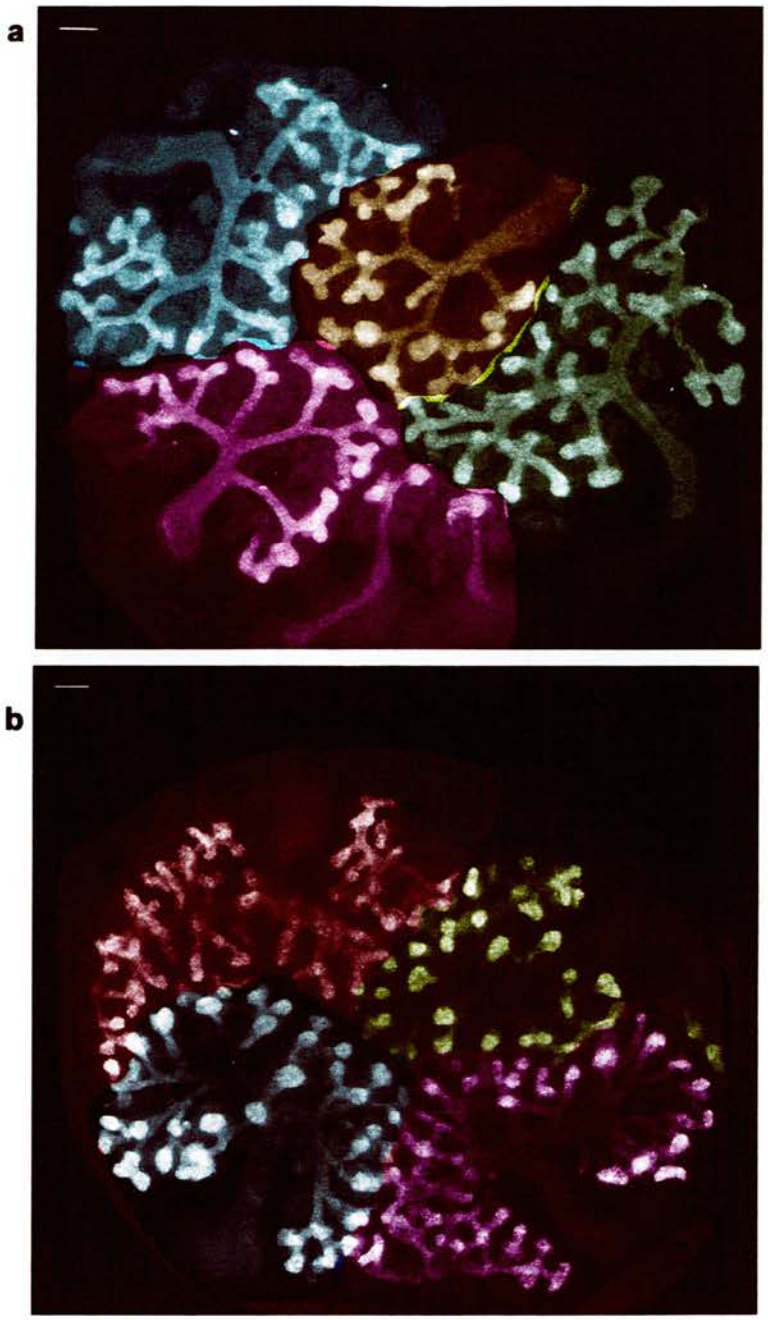


Figure 5.4: Anti-calbindin-D_{28k} staining of a cluster of E11.5 kidneys grown for 120hr and 168hr.

Kidneys were set up in a head to head configuration and grown for 120hr (a) or 168hr (b) before staining with anti-calbindin-D_{28k}. The image was converted to greyscale and was artificially coloured using Adobe Photoshop to distinguish between the 4 epithelial trees. Scale bar =100μm

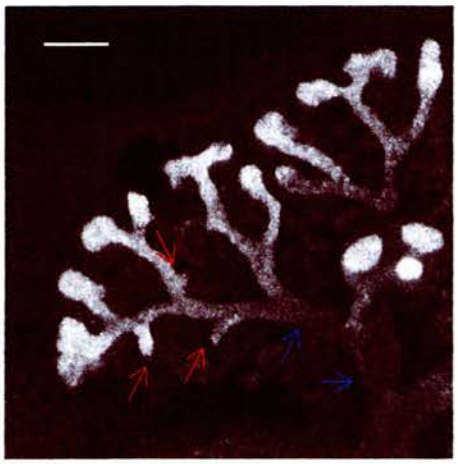


Figure 5.5: Staining of connecting ducts by anti-calbindin-D_{28k}

Above is an image of a 168hr clustered kidney stained with anti-calbindin-D_{28k} and putative connecting ducts are pointed out by the red arrows. Staining of connecting ducts by anti-calbindin-D_{28k} was obvious in some kidneys cultured for 120hr or 168hr in cluster or in isolation. The connecting ducts were identified as they were more tapered than the tips and sometimes the attached nephrons could be seen due to background staining. The proximal collecting ducts (blue arrow) show weaker staining for calbindin-D_{28k} than the distal ducts. The image was converted to greyscale and levels were adjusted using Adobe Photoshop Scale bar =100μm.

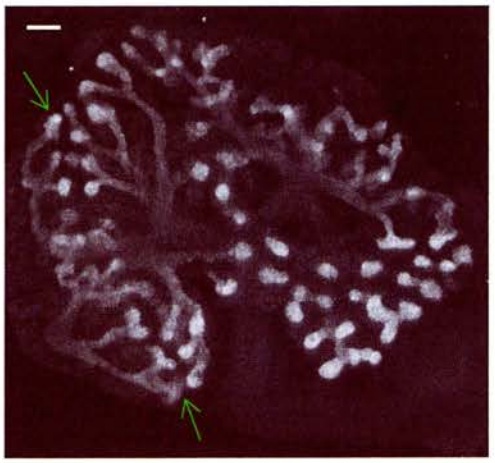


Figure 5.6: Staining of collecting ducts by anti-calbindin-D_{28k}

Above is an image of a 168hr clustered kidney stained with anti-calbindin-D_{28k}. Sometimes the collecting duct tips of the developing kidneys (both isolated and clustered) seemed to be directed towards the core of the kidney instead of towards the periphery (green arrow). The image was converted to greyscale and levels were adjusted. Scale bar =100μm.

5.3.2 Analysis of the number of tips per kidney in cluster and for kidneys grown in isolation

At first glance the kidneys in clusters did not appear obviously under or over branched. All kidneys had undergone multiple rounds of branching morphogenesis. In some instances tips were seen to come so close together that it was difficult to know where one ended and another began. In places it often appeared that tips of the kidneys in cluster did not avoid each other but seemed to be attracted towards each other. The kidneys did co-mingle somewhat, making the borders difficult to distinguish however the kidneys did not seem to grow far past or over each other suggesting that borders between kidneys were being observed.

Kidneys were placed on a collision course by clustering them together to see if they would react in any way to an unusually close neighbour. If the tips of a kidney 'sense' there are too many tips close by they might react by ceasing branching. To test this hypothesis the number of tips per kidney was counted for kidneys in cluster and kidneys in isolation (figure 5.7).

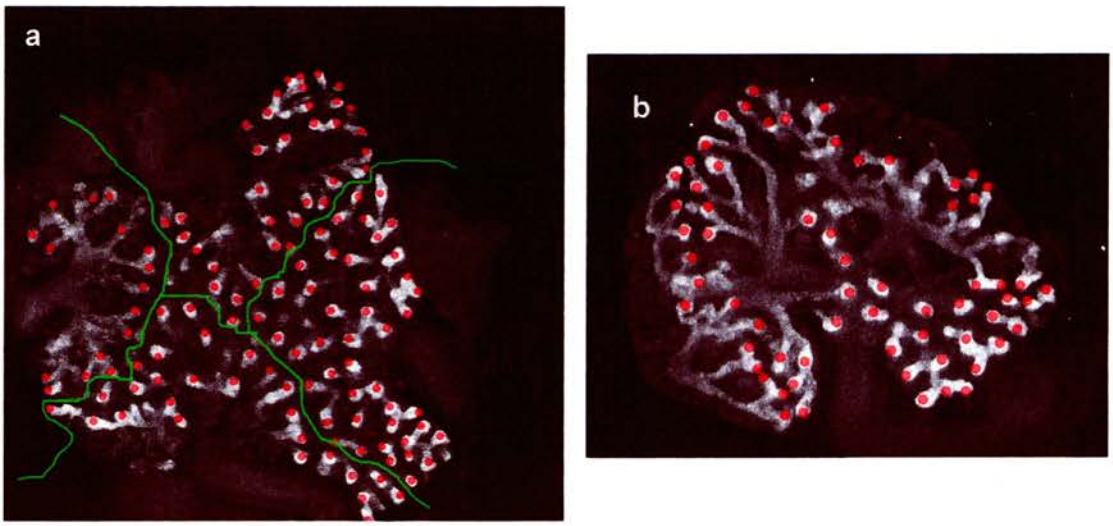


Figure 5.7: Counting the number of tips per kidney.

This image shows a 168hr cultured kidney cluster (a) and isolated kidney (b). They were fixed and stained with anti-calbindin-D_{28k}. The image was converted to greyscale and levels were adjusted using Adobe Photoshop. To aid counting the number of tips per kidney the tips were labelled with red dots and the boarders of the four kidneys in the cluster were estimated and outlined (green lines).

Comparisons were carried out to see if the kidneys in clusters differed in the number of tips they produced compared to controls (kidneys in isolation) at both 120hr and 168hr (figure 5.8). Kidneys in clusters were significantly under branched compared to isolated kidneys at both 120hr and 168hr of culture. It seemed that branching morphogenesis was halted when the kidneys were clustered as there was not a significant difference in the number of tips between 120hr and 168hr of culture. However, there was a significant difference between control kidneys from 120hr and 168hr of culture suggesting branching morphogenesis was still occurring in isolated kidneys.

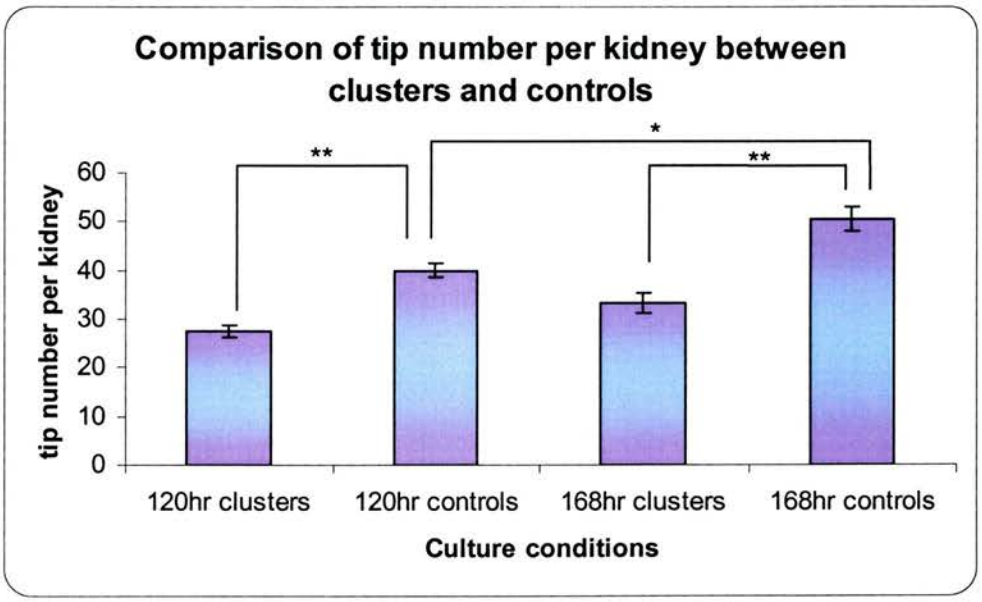


Figure 5.8: Comparison of tip number per kidney for kidneys in cluster or in isolation (controls) for 120hr and 168hr.

Tips per kidney were counted after culture in isolation or in clusters for 120hr or 168hr. Bars represent the mean number of tips per kidney on untransformed data. Significant differences between culture conditions were found ($F_{3,173}$=29, p<0.001) and significant differences between groups are indicated as follows, * p< 0.05, ** p<0.001. The natural log transformation was used for the statistical analysis in order to meet the assumptions of normality and homogeneity of variances. Bars = mean of 44 kidneys $^{+}$/- SEM.

5.3.3 Analysis of the eccentricity of the kidneys in clusters compared to the kidneys grown in isolation.

A kidney grown in culture flattens out and the ureteric bud branches radiate outward. The outline produced by the extremities of the ureteric bud can be thought of as an ellipse. If the ureteric bud branches grow and space out equally in all directions then a more circular ellipse is formed but if the branches grow further in one direction than the other then a more flattened ellipse results.

If the decision of a tip to grow is set by the rule 'navigate away if you are too close to another tip', then the kidneys in cluster may have an altered branching pattern compared to controls. To test this hypothesis, the eccentricity of the branching pattern produced by each kidney was measured and the average eccentricity was compared between kidneys in cluster and kidneys in isolation.

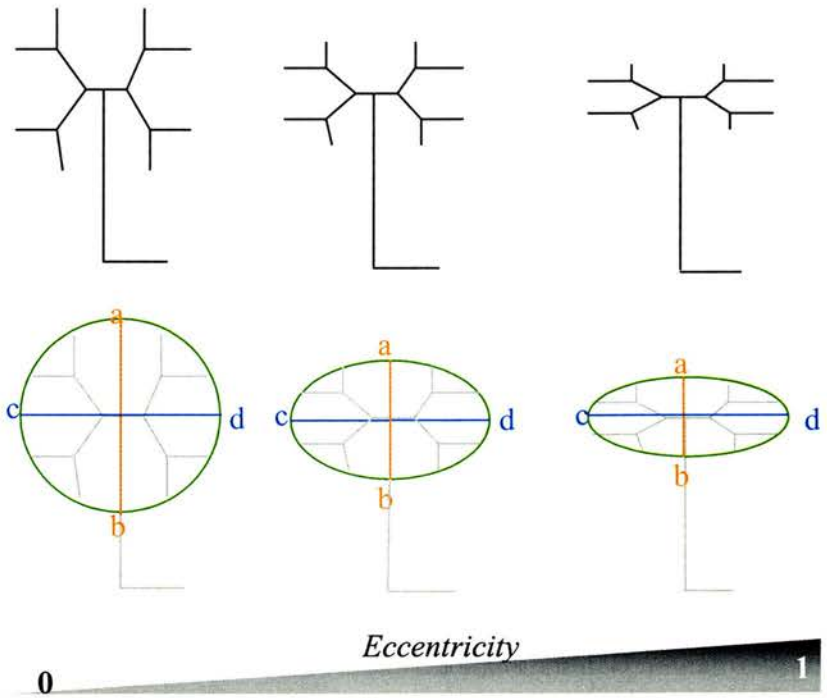


Figure 5.9: Illustration of eccentricity

Eccentricity, e, is a measure of how much an elliptic form deviates from a circle.

$$e = \sqrt{(1-(|ab|/|cd|)^2)},$$

where $(|ab|/|cd|)$ = minimum axis/maximum axis. $0 < e < 1$.

Therefore, as the previous illustration suggests (figure 5.9), the more similar an ellipse is to a circle the closer to 0 its eccentricity will be and the more deviant an ellipse is from circle the closer its eccentricity will be to 1.

The eccentricity of kidneys grown in clusters and in isolation for 120hr and 168hr was measured using Scion image processing software (figure 5.10). The peripheral outline of the ureteric bud was marked and the software calculated the minimum and maximum axes of the best fit ellipse.

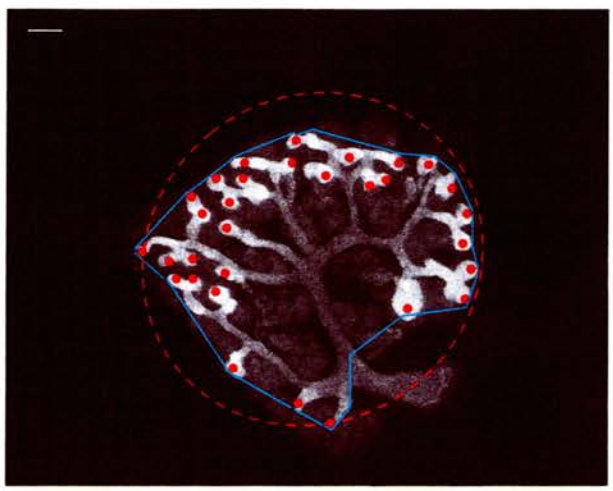


Figure 5.10: Demonstration of eccentricity measurement.

Scion image processing software was used to outline the peripheral outline of the kidney (blue line). Based on this, the program calculates the minimum and maximum axis of the best fit ellipse (as suggested by the red line).

The eccentricities of kidneys in cluster and in isolation were compared after 120hr and 168hr in organ culture (figure 5.11). There was significant difference in eccentricity between kidneys in cluster and those in isolation after 120hr of culture and also after 168hr of culture.

There was no difference between kidneys grown in cluster over time and similarly there was no significant difference found between kidneys grown in isolation over time.

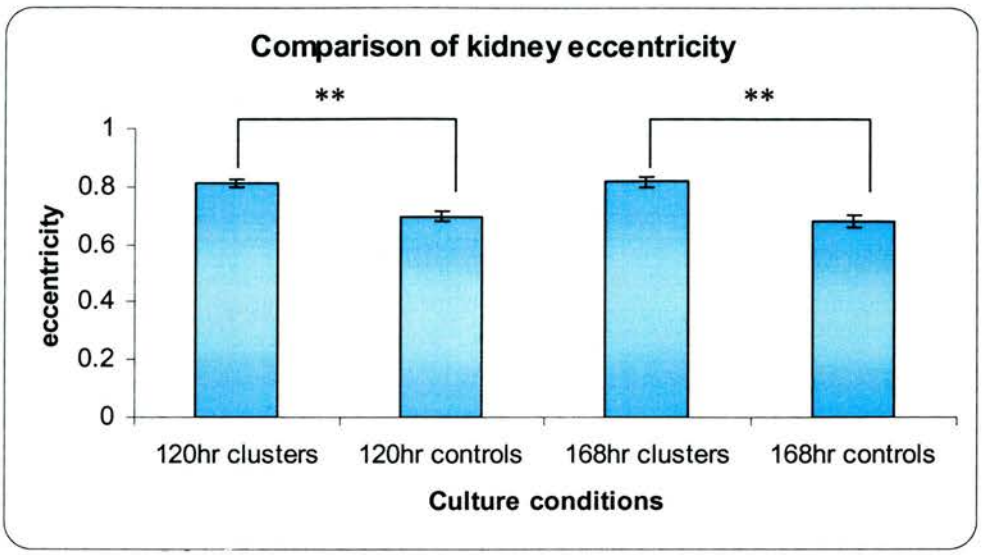


Figure 5.11: Comparison of eccentricity of kidney for kidneys in cluster or in isolation (controls) for 120hr and 168hr.

The eccentricity of each kidney in a cluster or in isolation (controls) was calculated after 120hr or 168hr of culture. Bars represent the mean eccentricity per kidney on untransformed data. Significant differences between culture conditions were found ($F_{3,173}$=21, p<0.001) and significant differences between groups are indicated as follows, * p< 0.05, ** p<0.001. The cubed transformation was used for statistical analysis in order to meet the assumptions of normality and homogeneity of variances. Bars = mean of 44 kidneys $^{+}$/- SEM.

The above eccentricity suggest that the kidneys in cluster are somewhat compressed compared to the control kidneys.

However, in order to quantify that this compression is taking place at the interface with a colliding kidney another analytical approach was used (figure 5.12).

Firstly, for the isolated kidneys

- the longest and shortest distance from the 1st branch point to the periphery was measured.
- Based on these measurements the average longest/shortest distance ratio for an isolated kidney was calculated.

Similarly for each clustered kidney

- The longest and shortest distance from 1st branch point to where it meets a neighbouring kidney was measured.

- And based on these measurements the average longest/shortest distance ratio for a clustered kidney was calculated.

The longest/shortest distance ratio was compared for isolated versus clustered kidneys. The hypotheses tested in this comparison were as follows.

The null hypothesis:
There is no difference between the longest/shortest distance ratio for kidneys in isolation and kidneys in cluster. This would indicate that kidneys in cluster are not compressed compared to controls.

The alternative hypothesis:
There is a difference between the longest/shortest distance ratio for kidneys in isolation and kidneys in cluster. If the ratio for the clustered kidneys is larger than the ratio from the isolated kidneys it would suggest that the clustered kidneys are not growing as far as expected in the direction of the neighboring kidney.

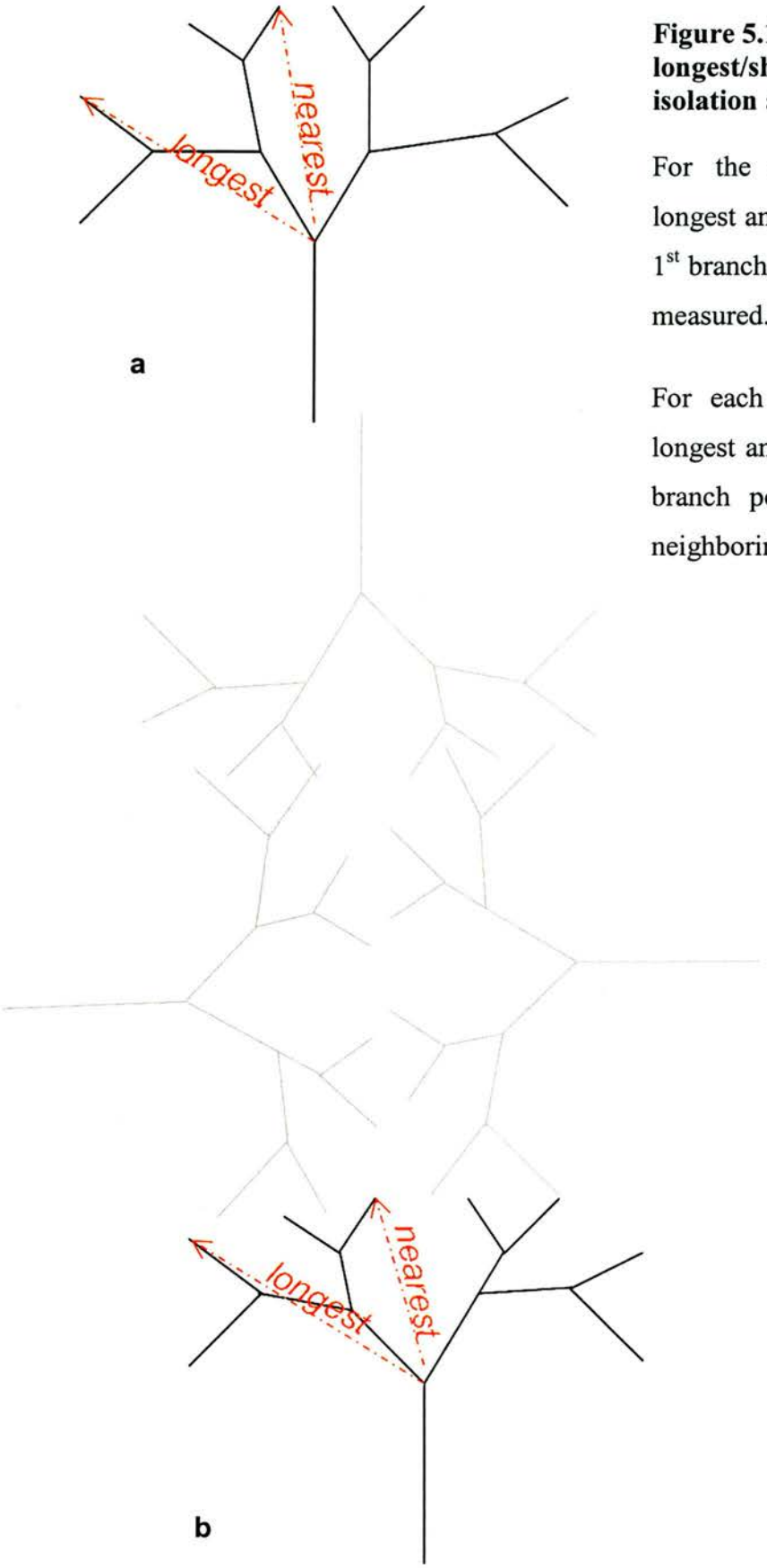


Figure 5.12: The measurement of longest/shortest ratio for kidneys in isolation and kidneys in cluster.

For the isolated kidneys (a), the longest and shortest distance from the 1st branch point to the periphery was measured.

For each clustered kidney (b), the longest and shortest distance from 1st branch point to where it meets a neighboring kidney was measured.

For either the kidneys in culture or in isolation the initial branching event was subjectively chosen if lateral branches supposed to have arisen from the stalk region of the ureteric bud were evident. Kidneys where the initial branch point was not obvious were discounted. The periphery of a kidney both in cluster and in isolation was deemed to be the limits of the kidney forward from the initial branching event (figure 5.12 and 5.13). In this way the distances to the tips which were coursing backwards were ignored.

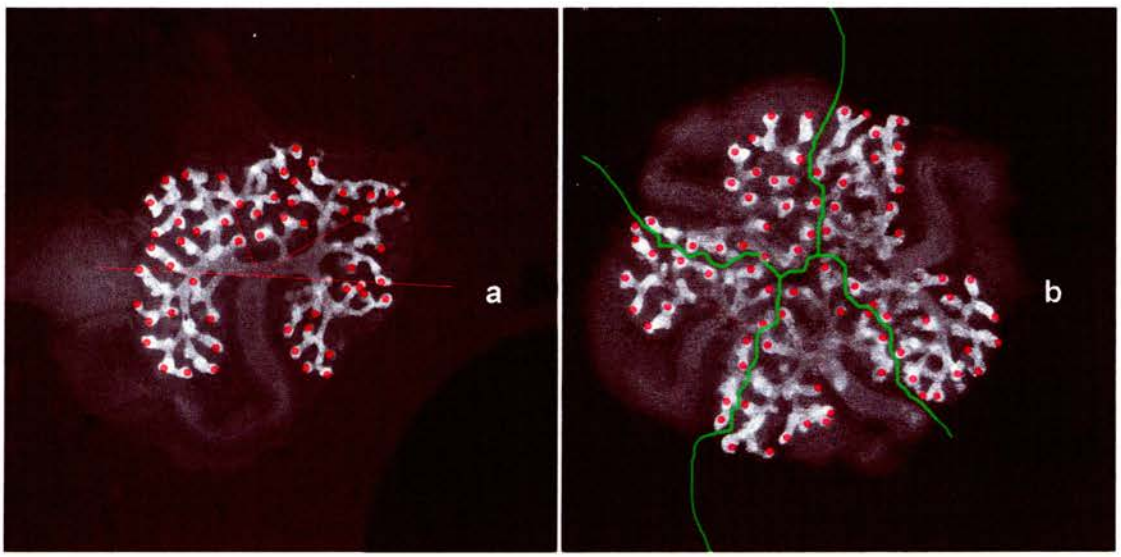


Figure 5.13: Measurement of the longest distance to the periphery and shortest distance from the initial branching event.

The longest distance and shortest distance forward from the initial branching event, was measured for both control kidneys (a) and kidneys in cluster (b). For the kidneys in cluster the shortest distance was always measured to the periphery where it collided with a neighbouring kidney (suggested by the green lines in b). For control kidneys the shortest distance to the periphery was measured instead.

The ratio of long/short ratio for control kidneys was compared to the long/short ratio for colliding kidneys.

There was significant difference between the ratios between 120hr clusters and controls (figure 5.14) although there was not enough evidence to suggest that there was a significant difference between the ratios between 168hr cultured clusters and

controls. The clustered kidneys showed a greater long/short ratio compared to the control kidneys at both 168hr and 120hr time points although only the 120hr groups showed statistically significant difference.

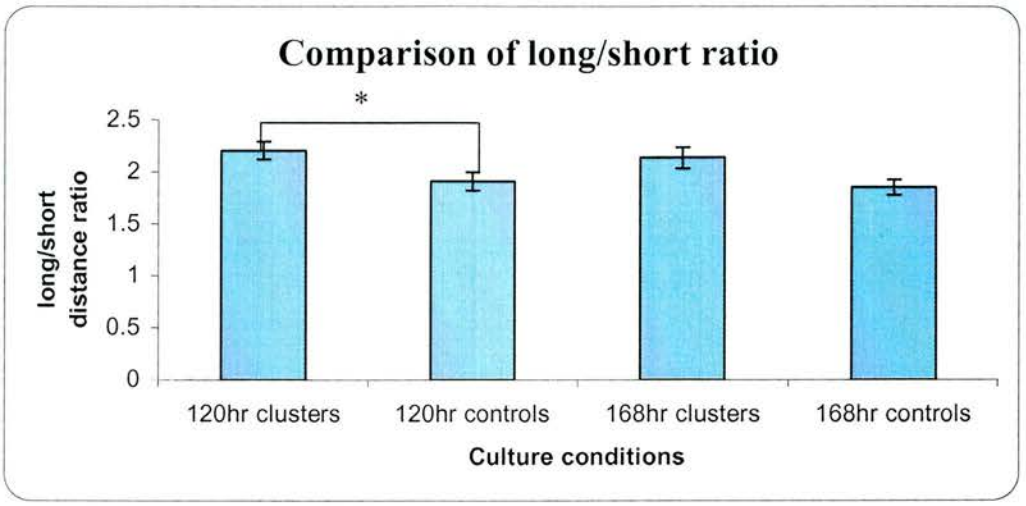


Figure 5.14: Comparison of long/short ratio from 1st branch point to periphery between clusters and control kidneys.

The long/short ratio was calculated for clustered kidneys and control kidneys after 120hr and 168hr of culture. Bars represent the mean long/short ratio per kidney on untransformed data. Significant differences between culture conditions were found ($F_{3,139}$=4.51, p=0.005) and significant differences between groups are indicated as follows, * $p<$ 0.05, ** $p<$0.001. The inverse transformation was used for the statistical analysis in order to meet the assumptions of normality and homogeneity of variances. Bars = mean of 41 kidneys $^{+}$/- SEM.

The long/short ratio for clustered kidneys is higher than the long/short ratio for control kidneys at least at 120hr. This higher long/short collision ratio of the clusters implies that the largest distance to the periphery of a clustered kidney predicts a smaller shortest distance than expected for an isolated kidney cultured for the same length of time. This suggests that, at least after 120hr of culture in clusters, kidneys are compressed in the direction of collision with a neighbouring kidney.

5.3.4 Analysis of the position of peripheral tips in one kidney relative to the tips of a neighbouring kidney

Given that I obtained this low-resolution evidence that tips of the ureteric bud may be prevented from approaching one another, I went on to investigate the spacing of tips along the collision interface at higher resolution.

I aimed to test whether the tips at the periphery of the kidney in a cluster are spacing out in an organised or in a random fashion in relation to the tips of the opposing kidney (figure 5.15). It is possible that the relative position of the tips of one kidney to an opposing kidney's tips is completely random or is following some pattern (maybe intercalated positioning or directly opposing positioning).

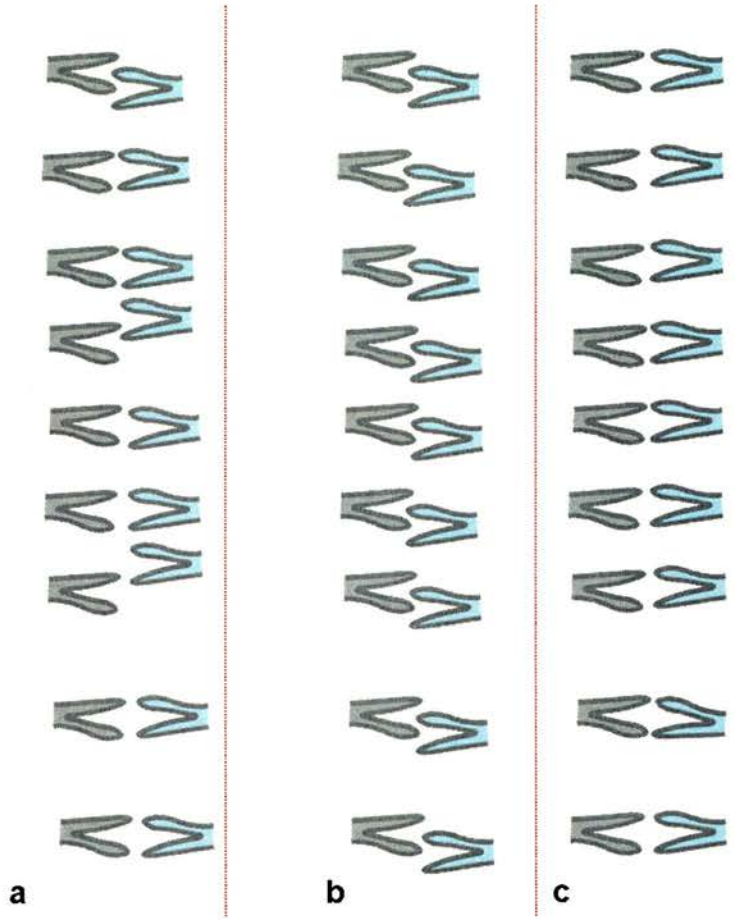


Figure 5.15: The relative position of peripheral tips.

Peripheral tips of kidneys in a cluster may or may not be randomly positioned relative to the opposing tips. Tips may space out randomly (as in a) relative to the opposite kidney. If tips do not space out randomly then they could have one of two arrangements; tips may intercalate with each other (as in b) or they may directly oppose each other (as in c).

The position of a tip relative to the opposite tips can be analysed as follows. Firstly, kidney A is chosen to assess the relative position of the tips of the opposing kidney, kidney B. Each neighbouring pair of peripheral tips of kidney A are used to define a plane which projects perpendicularly towards kidney B. This plane can be divided into three equal 'bins' (figure 5.16). A medial bin lies in-between the two tips while two lateral bins lie either side of the medial bin. The tip of kidney B may lie in the medial or lateral bin of the plane projected from the tips of kidney A (figure 5.17).

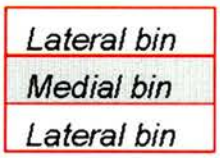


Figure 5.16: Plane of projection

The plane projected perpendicularly from a pair of peripheral tips of one kidney can be divided into three equal 'bins', two lateral bins which surround a medial bin.

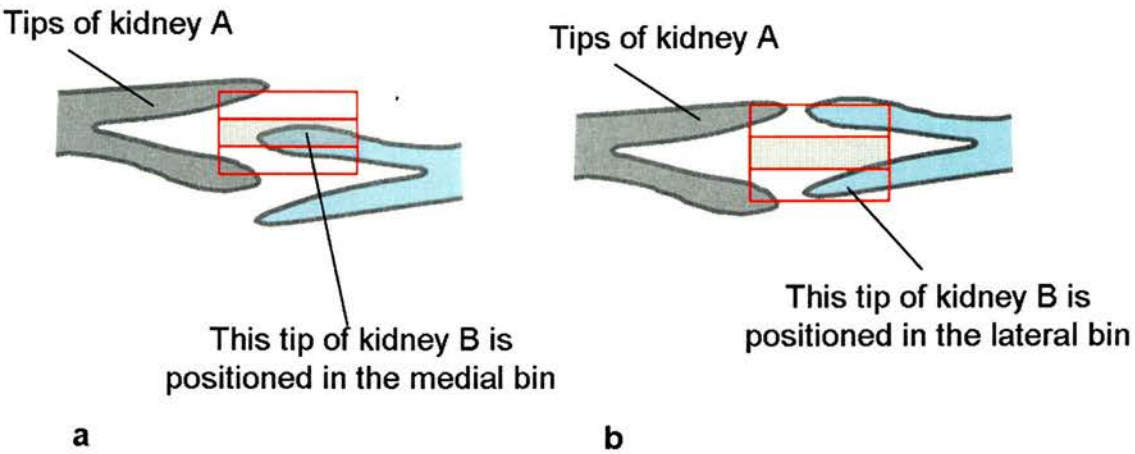


Figure 5.17: The position of tips within a plane projected from the opposite kidney.

A plane projected (red lines) from a pair of tips in kidney A is divided into three bins, two lateral bins and a medial bin (the medial bin is shaded in grey). The position of each tip in kidney B is then scored as to whether they are positioned in the medial bin (a) or in the lateral bin (b).

If the tips of kidney B are intercalating with the tips of kidney A then all of the tips of kidney B will lie in medial bins. If the tips of kidney B are directly opposing the

tips of kidney A then the tips of kidney B will lie in the lateral bins. If the tips of kidney B are randomly positioned relative to kidney A then there is a 33.3% chance that a tip of kidney B will be in the medial bin (similarly there is also a 33.3% chance that a tip of kidney B will be in either of the lateral bins).

After counting the number of tips of kidney B which lie in medial or lateral bins relative to kidney A the reverse can be carried out (figure 5.18). This time the relative position of tips of kidney A are analysed with respect to the tips of kidney B. A plane is projected from each neighbouring pair of peripheral tips of kidney B. Similarly this plane is divided into three equal bins, two lateral and one medial bin. The tips of kidney A are then scored as to whether they line in a lateral or in the medial bin of the plane projected from kidney B.

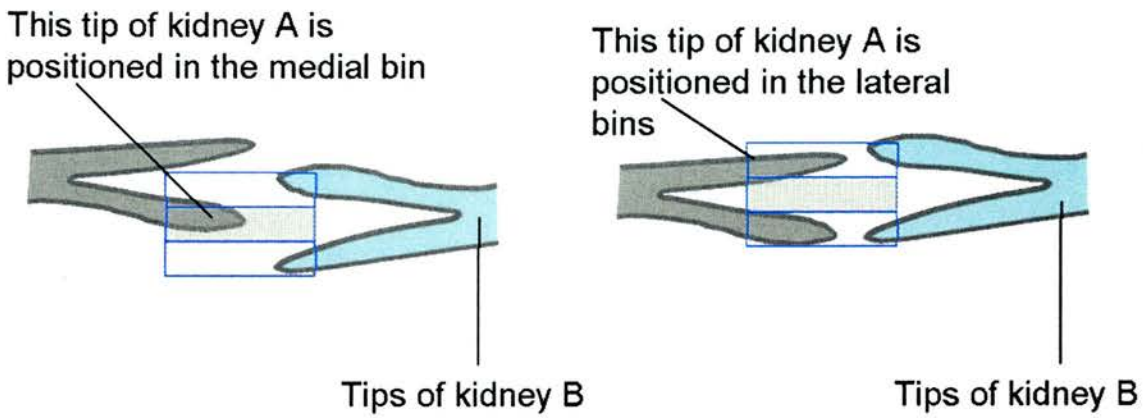


Figure 5.18: The position of tips within a plane projected from the opposite kidney.

A plane projected (blue lines) from a pair of tips in kidney B defined an area which is subdivided into three equal bins, two lateral bins and a medial bin (the medial bin is shaded in grey). The position of each tip in kidney A is then scored as to whether they are positioned in the medial bin (a) or in the lateral bin (b).

In this way the peripheral tips of each kidney in a cluster is scored as lying in a lateral or medial bin (figure 5.19). If the tips are randomly spacing out relative to each other then only 33.3% of the tips will be positioned in a medial bin. If the tips of the kidneys are intercalating with each other then each peripheral tip will lie in a

medial bin. If the tips are directly opposing one another then they will lie in a lateral bins and none will lie in medial bins.

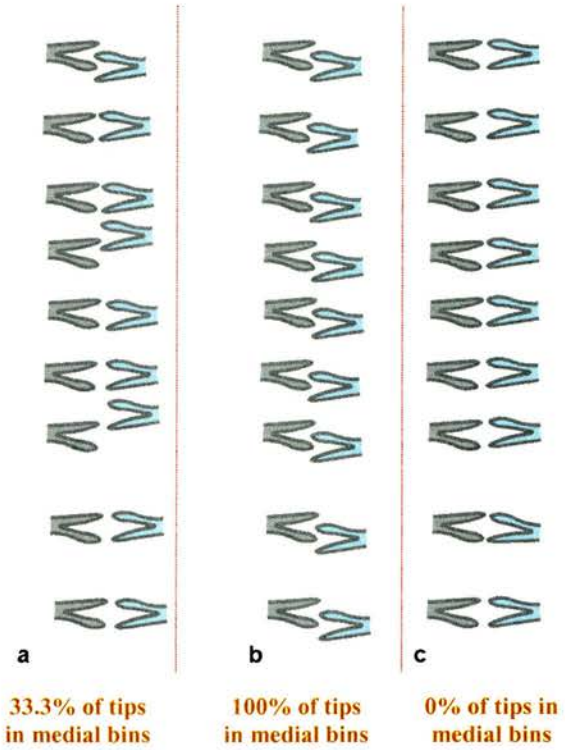


Figure 5.19: Scoring the relative position of peripheral tips.

If the peripheral tips of kidneys are randomly positioned relative to an opposing kidney then ideally 33.3% of all the tips should lie in the medial bin of a plane projected from opposing tips (a). However if tips intercalate with each other then 100% of the tips scored should lie in a medial bin (b). If tips directly oppose each other then 100% of the tips should be lying in lateral bins but no tips should be lying in medial bins (c).

The analysis outlined above was applied to the 120hr and 168hr cultured kidneys. The peripheral tips were subjectively labelled for each kidney in a cluster (figure 5.20). Based on the planes projected from each pair of tips towards its neighbouring kidney, tips were scored as to whether they were positioned medially or laterally relative to the opposing nearest tip (figure 5.21).

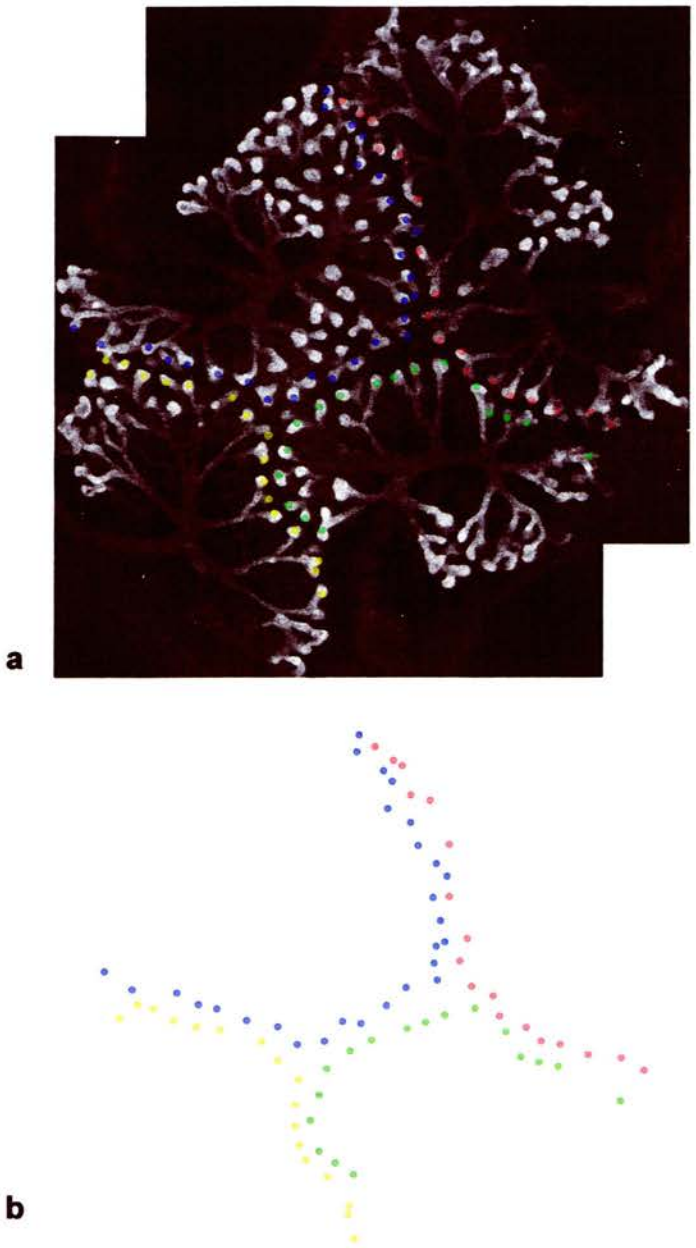


Figure 5.20: Labelling peripheral tips of kidneys in a cluster.

This cluster of kidneys was cultured for 168hr and the tips at the periphery of each kidney were marked using Adobe Photoshop (a). A different colour was used to mark the tips of each kidney. The image layer (b) with the marked peripheral tips was saved for later use to scoring the relative position of the tips.

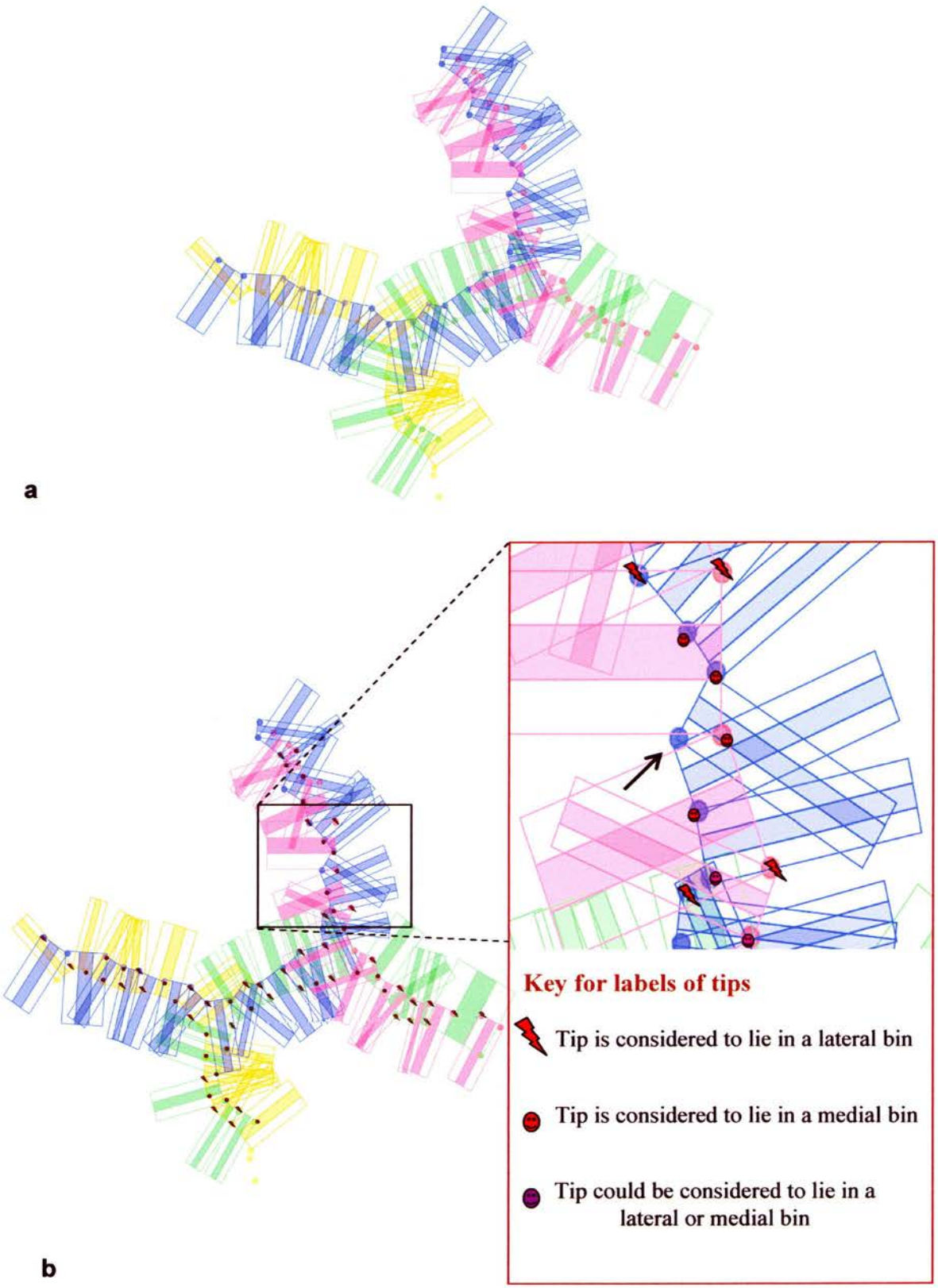

a
b
Key for labels of tips
Tip is considered to lie in a lateral bin
Tip is considered to lie in a medial bin
Tip could be considered to lie in a lateral or medial bin

Figure 5.21

Figure 5.21: Scoring the peripheral tips of kidneys in a cluster.

Planes were projected from each set of neighbouring tips of each kidney using Microsoft PowerPoint (a) (blue, green, yellow and pink all represent the set of tips from one of the four kidneys in a cluster). Each tip was then scored as to whether it was positioned in the medial or lateral bin of the planes projected from the nearest tip of the opposing kidney (b). A zoomed in view of a section of tips which have been scored is also shown in figure b. The key for the labels used to score the tips is also presented. Tips which do not occupy a bin are not counted (arrow in figure b). Tips which can either be considered to lie in a medial or lateral bin are noted separately.

This analysis was carried out for 120hr and 168hr kidney clusters. Table 5.1 summarises the results:

Clusters	Tips in medial bins	Tips in lateral bins	Tips which can be considered in a medial or lateral bin
120hr	130 (37%)	213 (61%)	9 (3%)
168hr	152 (36%)	241 (57%)	31 (7%)

Table 5.1: Table of results for the relative position of tips to their nearest opposing neighbour.

The tips which lie in a medial and lateral bin can be treated either as lateral tip or medial tips. If the tips that can be considered lateral or medial are treated as being positioned in a medial bin, the results table changes to (table 5.2):

Clusters	Tips in medial bins	Tips in lateral bins
120hr	139 (39%)	213 (61%)
168hr	183 (43%)	241 (57%)

Table 5.2: Table of results for the relative position of tips to their nearest opposing neighbour if the tips that can be considered lateral or medial are treated as being positioned in a medial bin.

If the tips that can be considered lateral or medial are treated as being positioned in a lateral bin, the results table changes to (table 5.3):

Clusters	Tips in medial bins	Tips in lateral bins
120hr	130 (37%)	222 (63%)
168hr	152 (36%)	272 (64%)

Table 5.3: Table of results for the relative position of tips to their nearest opposing neighbour if the tips that can be considered lateral or medial are treated as being positioned in a lateral bin.

To test whether these results agree with the theory that the tips at the periphery of a cluster are randomly distributed with respect to each other the following hypothesis were defined:

H_0: The null hypothesis: the proportion of tips in medial bins is equal to 33.33%, the proportion predicted if tips were randomly positioned.
H_1: The alternative hypothesis: The proportion of tips in medial bins is not equal to 33.33%, the proportion predicted if tips were non- randomly positioned.

Statistical comparisons were carried out using Microsoft Excel. Each binomial distribution is defined by the properties:

N = the total number of tips investigated

k = the number of tips observed to lie in a medial bin

p = the probability that each tip will lie in a medial bin

q = the probability that each tip will not lie in a medial bin

$\mu = N \times p$ = the mean

$\sigma = \sqrt{(N \times p \times q)}$ = the standard deviation

Each binomial distribution was converted to the standard normal distribution using the formula

$$z = \text{standard deviations} = \frac{(k - \mu) \pm 0.5}{\sigma}$$

For each value of k, a z value was obtained and the corresponding probability value could be found in mathematical tables (I used the online z to p calculator for my analysis. It is located at the URL http://faculty.vassar.edu/lowry/ch6px.html. The following data tables summarise the resultant p values.

If the tips that can be considered lateral or medial are treated as being positioned in a medial bin, the results are as follows (table 5.4):

Clusters	Tips in medial bins	Tips in lateral bins
120hr	139 z=2.39 p=0.0085	213 (61%)
168hr	183 z=4.24 p<0.005	241 (57%)

Table 5.4: Result of a binomial test if the tips that can be considered lateral or medial are treated as being positioned in a medial bin.

As the p values are less than 0.05 the null hypothesis is rejected. It seems that if tips that can be considered to be placed in a lateral or medial bin are treated as medial bin tips that there is a greater tendency for the tips at the periphery of the kidneys to be positioned in a medial bin rather than to be positioned randomly relative to the nearest opposing tip.

If the tips that can be considered lateral or medial are treated as being positioned in a lateral bin, the results are as follows (table 5.5):

Clusters	Tips in medial bins	Tips in lateral bins
120hr	130 z=1.38 p=0.08	222 (63%)
168hr	152 z=1.05 p=0.15	272 (64%)

Table 5.5: Result of a binomial test if the tips that can be considered lateral or medial are treated as being positioned in a lateral bin.

Each p value is greater than 0.05 and so there is not convincing evidence to reject the null hypothesis. This scenario, of treating undefined tips as lying in lateral bins, is erring on the side of caution. If there was enough evidence to reject the null hypothesis when erring on the side of caution then it would not matter whether the undefined tips were classified as lateral or medial. There is not enough evidence to reject the null hypothesis when erring on the side of caution. Therefore it is still possible that the tips at the periphery of the kidneys in cluster are randomly positioned with respect to the closest opposing tip (when tips that are placed in medial and lateral bins are treated as lateral tips).

5.3.5 Analysis of the nearest neighbour distances between tips of clustered and isolated kidneys

Lastly I investigated whether the navigation of a tip is achieved by the rule 'if you are within a certain distance, $x\mu$m, to another tip, do not grow towards it (either stop growing or turn away)'. If this hypothesis is correct, it should not be possible for tips to approach closer than $x\mu$m microns even if placed on a collision course. To test this hypothesis the average 'nearest neighbour' distance between tips was measured and compared between kidneys in cluster and those in isolation after 120hr of culture (figure 5.22).

The tips of kidneys were labelled with red dots using Photoshop. Scion image software was then used to measure the closest distance from each tip to its nearest neighbour. Two rules were observed when carrying out the measurements:

- Firstly the nearest neighbour of a tip must not be a direct sister of the tip. If two tips share the same direct parent branch then they are not considered neighbours. It is obvious that sister tips will be positioned quite close to each other especially during early stages of branching.
- Nearest neighbour distances were recorded once. There were no double entries recorded. In other words if tip A's nearest neighbour is tip B by $x\mu$m but tip B's nearest neighbour is also tip A by $x\mu$m then the distance $x\mu$m is only recorded once.

The distances between tips for kidneys in clusters and kidneys in isolation was compiled and was presented as histogram distributions (figure 5.23, graph a and b). Data was also presented as percentage frequency distributions (figure 5.23, graph c, d and e). The 'nearest neighbour' distance between tips was significantly greater for kidneys grown in isolation compared to those grown in clusters (figure 5.23, graph f).

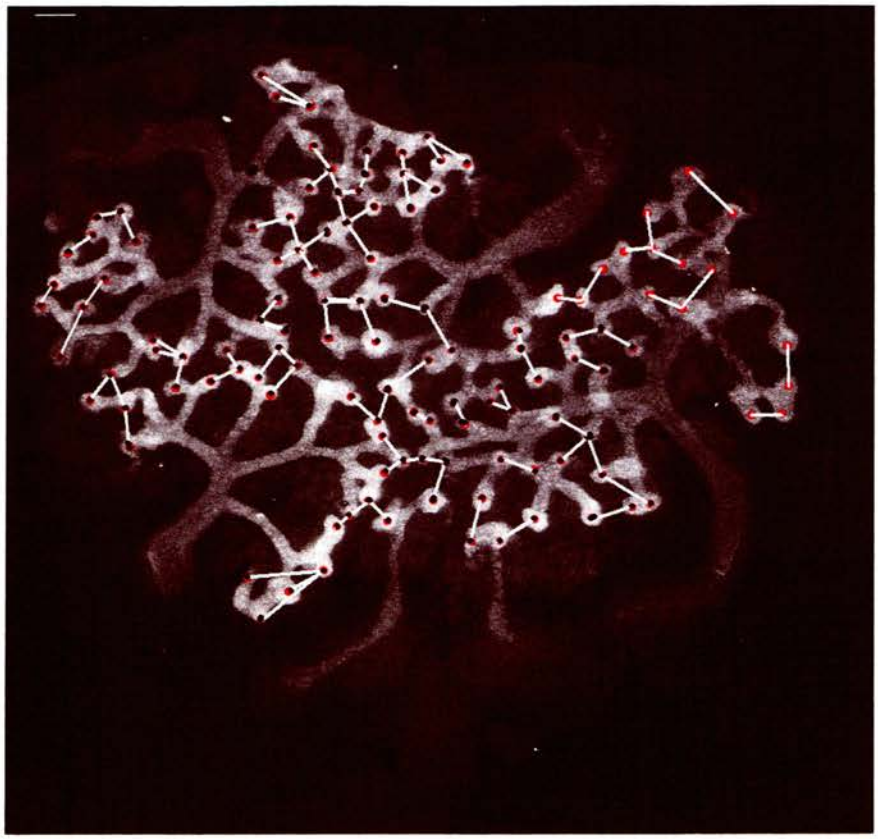


Figure 5.22: Demonstration of the measurement of nearest neighbour tip distances

The minimum tip distances between tips was measured for the tips of clustered kidney cultured for 120hr. Isolated kidneys were also analysed. The distance between tips was measured using Scion image processing software. Double distances were not counted and sister tips were not considered nearest neighbours.

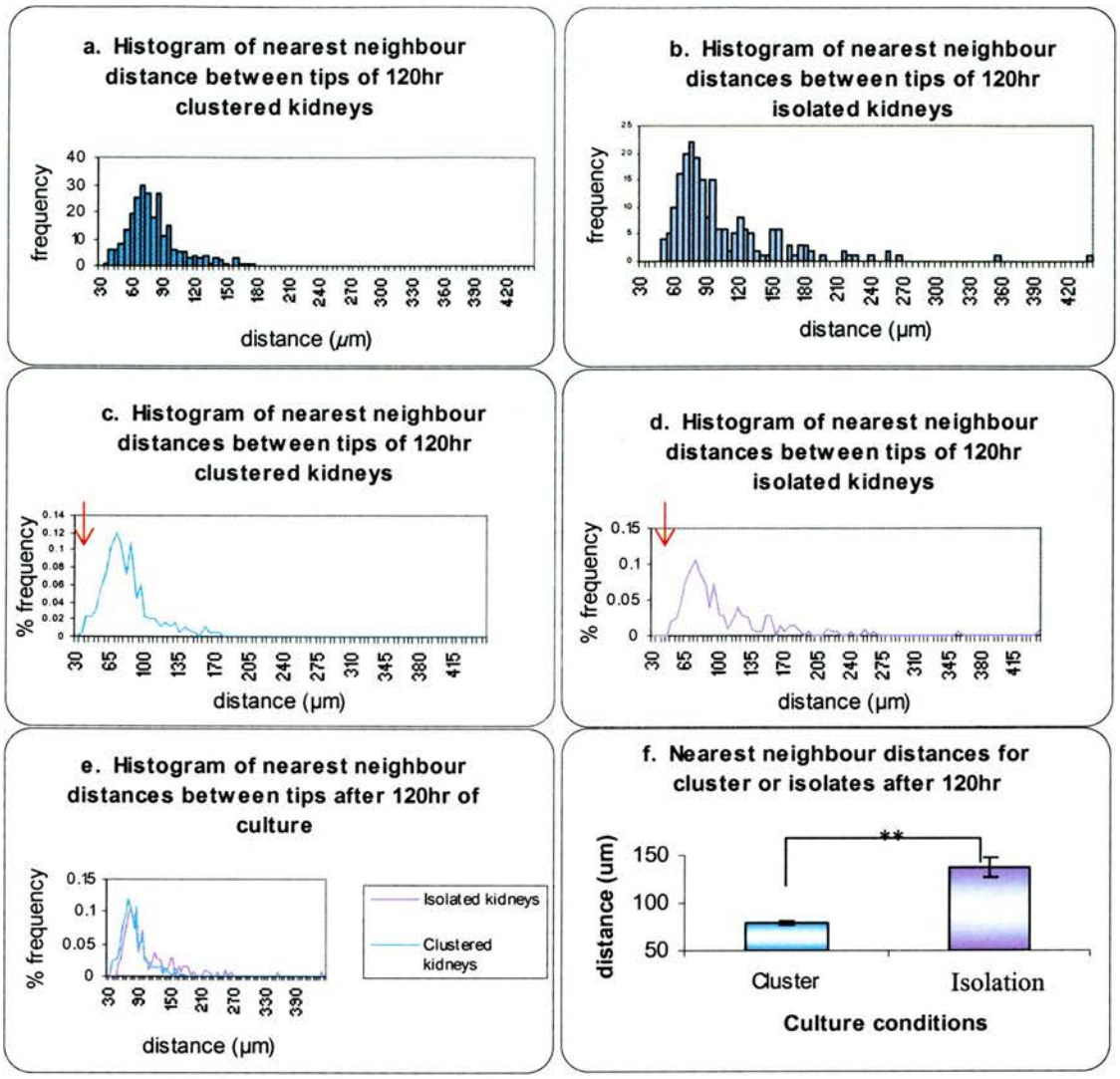


Figure 5.23: Analysis of nearest neighbour distances for 120hr cultured kidneys in clusters or in isolation.

Graphs a and b represent frequency distributions for the nearest neighbour distances between tips for kidneys cultured for 120hr in cluster (a) and in isolation (b). These data is also presented as percentage frequency graphs for clustered kidneys (c) and for kidneys in isolation (d) and the overlay is presented in (e). The red arrows in c and d highlight the minimum distance that these distributions are tending towards. Graph f illustrates the comparison between the nearest neighbour distances for kidneys in cluster or in isolation after 120hr of culture. Bars represent the mean distance between tips on untransformed data. Significant differences between culture conditions were found ($F_{1,468}$=39.90, p<0.001) and significant differences between groups are indicated as follows, * p< 0.05, ** p<0.001. The inverse square root transformation was used for the statistical analysis in order to meet the assumptions of normality and homogeneity of variances. Bars = mean of 211 tips $^{+}$/- SEM.

The nearest neighbour distance seems to approach a minimum distance (refer to figure 5.23, graph c and d). The red arrows in these graphs suggest the minimum distance

to which these graphs tend. It seems that the tips of the kidneys are navigating closer than is expected for an isolated kidney. However the measurements may be deceptive when considering the following. In some cases the tips of the kidneys come so close together that it is difficult to determine where one ends and the other begins. From a stack of confocal images it seemed that tips were colliding with each other in the clustered cultures but also sometimes in the isolated kidneys. One potential tip collision, found between two tips from different ureteric buds of a 120hr cluster culture, was investigated further. Thinner optical sections of 0.5μm were chosen to image the two tips using confocal microscopy (figure 5.24).

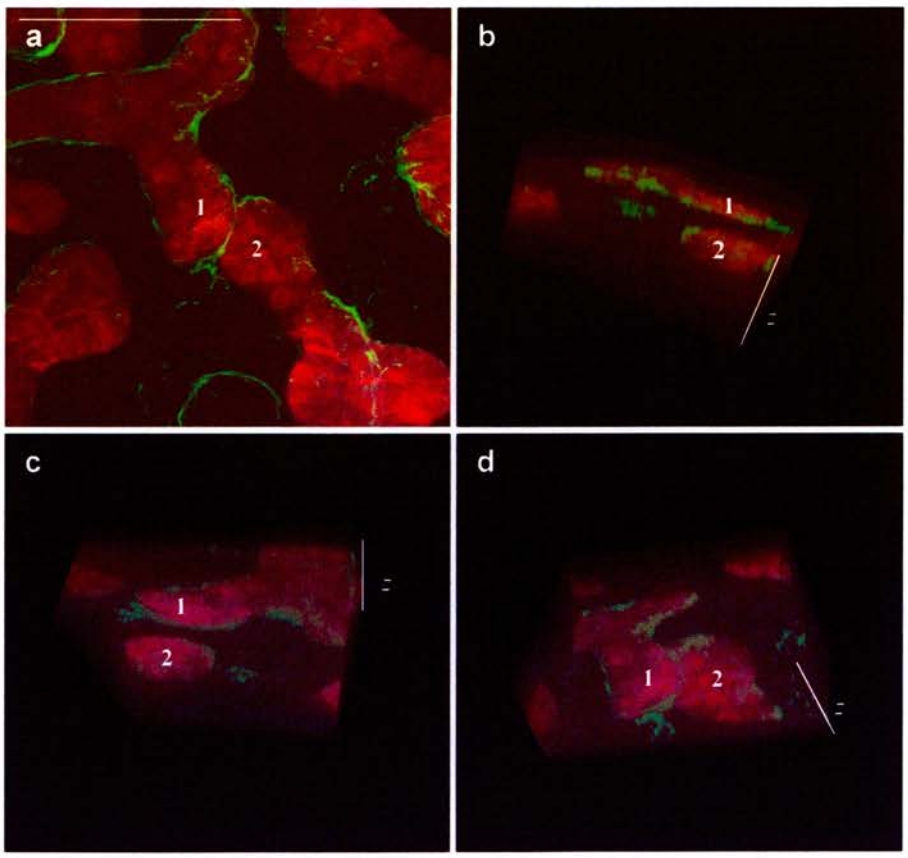


Figure 5.24: Analysis of tip collisions

Although 2D images of clustered kidneys suggested that tips from different kidneys (labelled 1 and 2) can collide with each other, the same image in 3D highlighted that the tips are in fact a distance apart from one another and are not in intimate contact. Figure a scale bar = 100μm. Depth, *z* of 3D images b-d = 57μm (step size of scan = 0.5μm). The 3D image was created using ImarisSurpass software with the assistance of Linda Wilson.

The above image demonstrates that there is a substantial distance between tips with respect to the thickness of the culture itself. Therefore the distance between tips from 2D images is not the actual distance between tips but is the 'width' measurement as illustrated below (figure 5.25). The actual distance between tips can only be calculated when the 'width' and 'depth' measurements are known. According to Pythagoras' theorem,

$$(\text{distance between tips})^2 = (\text{width}^2 + \text{depth}^2)^{½}$$

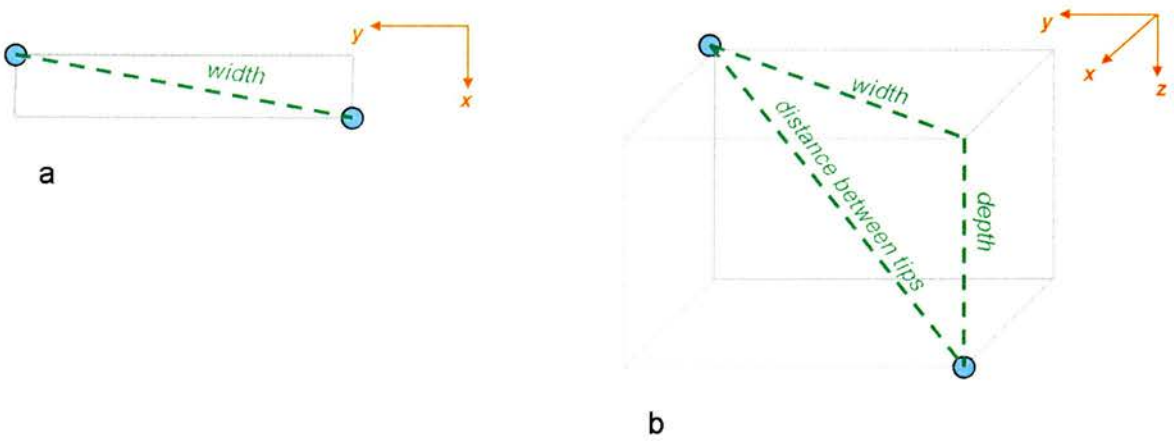


Figure 5.25: Consideration of the distance between tips in 3D.

From a 2D image (figure a) in which the cultured kidneys are viewed directly from above the distance between two tips (blue circles) appears to be the measurement 'width'. However when the 3D nature of the culture is considered (figure b) it is clear that there is an appreciable distance in depth between the neighbouring tips also. Therefore, when taking account of the 3D nature of the culture, the distance between tips can only be calculated using Pythagoras' theorem when the width and depth measurements are known.

From the optical serial sections of the 120hr cultured kidneys and clusters it was attempted to find the position of each tip within the depth of the culture i.e. to record *z*. However the scans of the clusters were unable to give this information. A typical 120hr kidney cultured (either cultured as an isolate or cluster) was imaged with optical sections taken every 5μm (figure 5.26). These sections were found to be too bulky and thick to determine accurately the depth at which each tip was situated within the culture. The majority of tips seemed to lie at the same depth. Therefore it was impossible to calculate the actual distance between tips using this approach.

Other methods of accurately measuring the nearest neighbour distance between tips would have been considered.

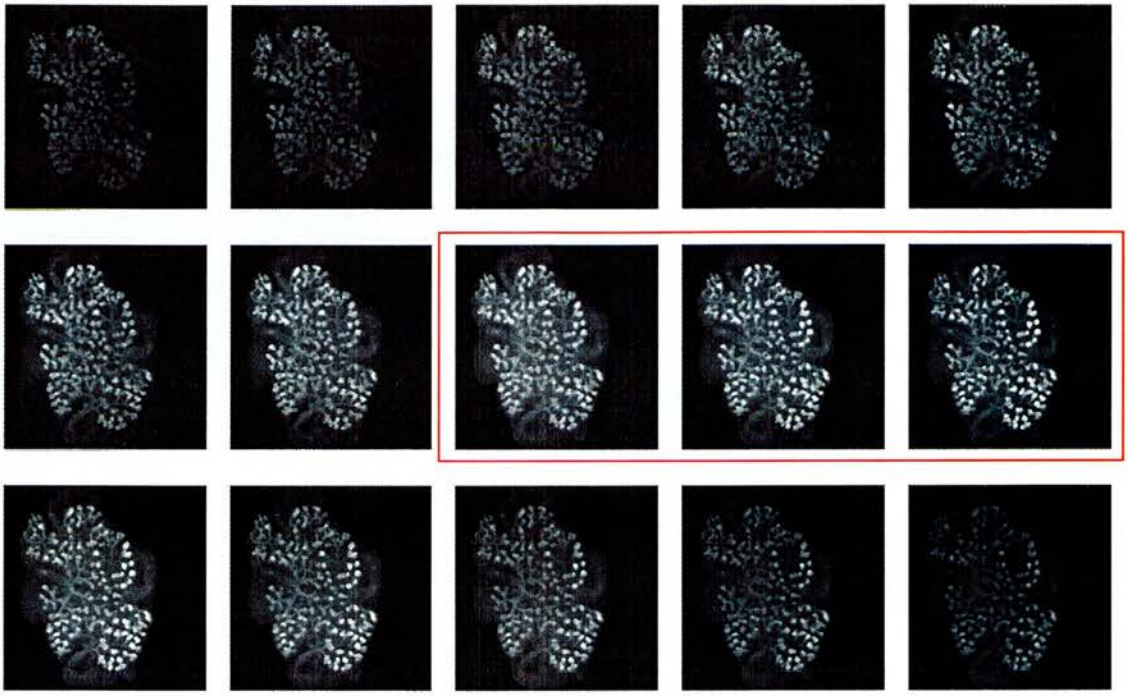


Figure 5.26: Images from an optical section of a cluster of 120hr cultured kidneys.

These images are 15 consecutive sections from 32 optical confocal sections (step size of 5μm) of a 120hr cluster of kidneys. It is difficult from these sections to decide the depth at which each tip lies within the thickness of the cluster and it appears that the majority of tips lie at the same depth (highlighted by red box).

On the whole it appears that when kidneys are clustered together they are under branched and have a distorted overall shape compared to kidneys cultured in isolation. The shortest distance from the 1st branch point to the periphery is lower than is expected compared to controls suggesting that the clustered kidneys are not growing as far forward as they should be towards the opposing kidneys, at least after 120hr of culture. There was not sufficent evidence to suggest that the peripheral tips of kidneys in cluster are non-randomly distributed relative to the tips of an apposing kidney. Unfortunately I was unable to determine whether the tips space out to observe a minimum distance between neighbouring tips during branching morphogenesis.

5.4 Discussion

The discussion focuses on the following:

- Space restrictions
- Comparative controls
- Collisions between tips
- A minimum distance between tips
- Anti-calbindin-D_{28k} staining
- Centripetal growth

The investigations presented in this chapter sought to examine the tip-tip interaction that may be important in shaping the branching pattern of the ureteric bud. To test this, kidneys were forced to collide with each other by clustering them in culture. The reaction of the kidneys and effects of the collision was analysed using a variety of methods. The ureteric bud trees were affected in a number of ways. In addition to being significantly under branched compared to kidneys grown in isolation, the clustered kidneys seemed to be compressed and their growth was retarded in places where they collided with a neighbouring kidneys. The kidneys did not seem to invade far into each other's territory. In addition to these findings however, there was not significant evidence to suggest that tips at the periphery of clustered kidneys to interdigitate with opposing kidney tips and so it is possible the tip positioning could be random. It was not possible to determine whether there was a minimum distance maintained between tips.

5.4.1 Space restrictions

Although it is possible that the under branched and abnormal patterns formed from the clustered kidneys are due to the direct effects of tip to tip signalling it must be considered that these results could also be due to other factors. Hypoplasia of other branching organs such as the lung can occur due to physical obstruction of the space into which it is growing, for example congenital diaphragmatic hernia can

result in extrusion of abdominal viscera into the plural cavities and thereby impede the growth of the developing lungs (Gosche *et al.* 2005; Rottier *et al.* 2005). Possible space restrictions at the interface of the developing kidneys when clustered together could disrupt expansion of the nephrogenic zone at the periphery, thereby having a knock-on effect on branching morphogenesis. Other restrictions on growth could include insufficient diffusion of nutrients to the centre of the kidneys. It has been suggested previously that the decrease in the rate of growth of kidneys in culture, which occurs after about 24hr, could be due to the restricted diffusion of nutrients into the expanding explant (Cullen-McEwen *et al.* 2002). Without a developing blood supply in organ culture the cells rely on diffusion of nutrients to support growth. Therefore the under branched nature of the clusters kidneys could be due to the reduced diffusion of nutrients.

5.4.2 Comparative controls

For many of the experiments presented in this chapter clustered kidneys were compared to kidneys grown in isolation. There are a number of limitations to the use of isolated kidneys as a control group. Isolated kidneys are assumed to be unimpeded in their growth. As mentioned above there may be an effect on kidney growth in cluster due to a non-specific restriction of space or nutrients. In order to account for such experimental effects it would be more insightful to use the following controls for comparison with clustered kidneys (figure 5.27).

- There may be effects on growth in the clustered kidneys due to insufficient space or nutrients caused by the enhanced tissue thickness of the kidney cluster itself. To account for this it would be useful to analyse the growth of a kidney surrounded by three additional kidney mesenchyme (figure 5.27b). In this manner the kidneys is exposed to a physical tissue barrier from the extra mesenchyme which should more appropriately mimic any effects seen on the growth of the clustered kidneys due to space/nutrient restrictions.

- It is possible also that the kidneys of a cluster are unable to grow into the neighbouring kidney mesenchyme because it has been induced by its own ureteric bud. To account for this, kidneys should be cultured with mesenchyme which has been previously induced by the heterologous inducer, dorsal spinal cord (figure 5.27c). In this way any restrictions on the growth of the kidneys in cluster due to their inability to penetrate induced mesenchyme would be highlighted.

It is likely that a combination of the aforementioned controls would be most helpful in understanding the influence of tip-tip interactions on the growth of kidneys in clusters. Therefore in order to make more concrete conclusions these controls are necessary.

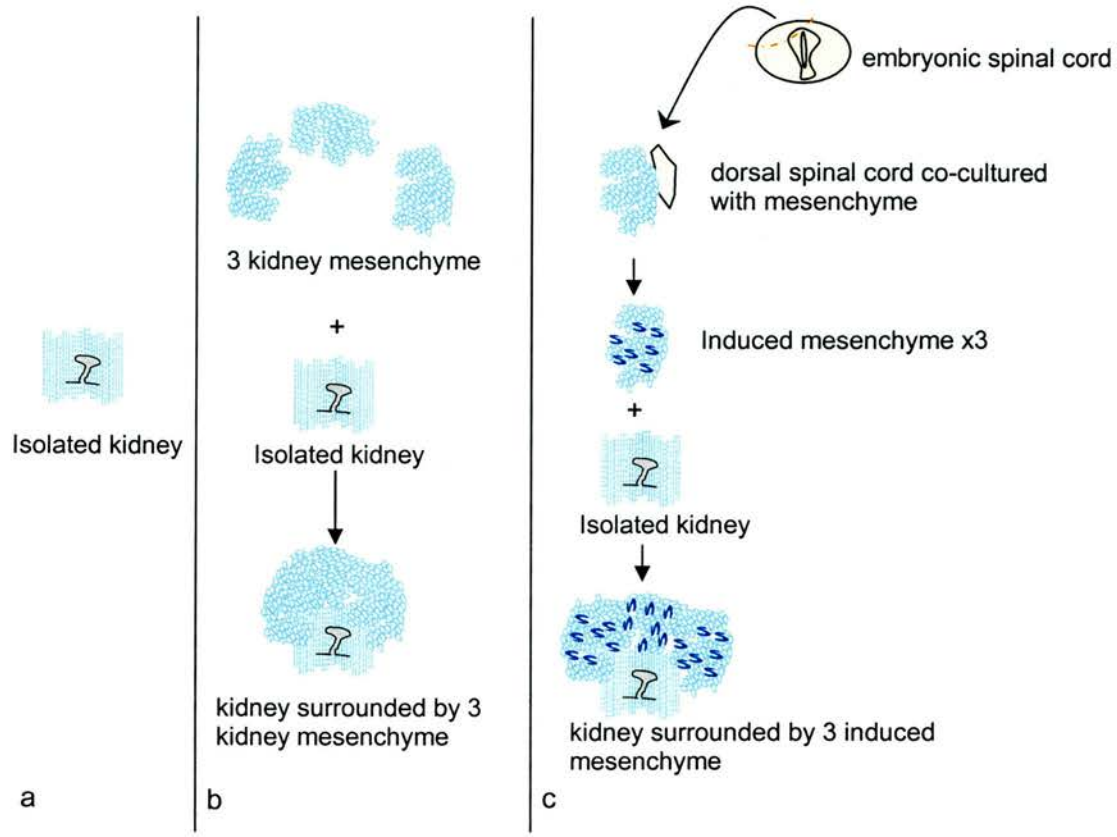


Figure 5.27: Comparative controls for kidneys in cluster

In order to fully understand the role that tip-tip interactions play in the growth retardations seen with cultured kidney it would be more insightful to use alternative comparative controls to that of an isolated kidney (a). To account for possible restrictions on growth due to space or nutrient limitations, a kidney surrounded by three mesenchymes should be analysed (b). In order to assertain whether the growth inhibitions of a clustered kidneys are due to the pre-induction of the neighbouring kidney's mesenchyme it would be of interest to analyse the growth of a kidney when surrounded by mesenchyme in which induction has been previously carried out by co-culture with dorsal spinal cord (c).

5.4.3 Collisions between tips

The data presented in these studies suggest that the clustered kidneys do not grow past each other and that the shortest distance to the collision is shorter than expected. This is compatible with the theory that tips of nearby kidneys avoid each other. In the clustered kidneys there was no obvious evidence of collisions or fusions

between tips. The lumen of the kidney is patent throughout the extent of the epithelial tree (Meyer *et al.* 2004) and microinjections of fluorescent molecules such as FITC-dextran sulfate into the lumen of the ureteric bud as carried out by Meyer et al. (Meyer *et al.* 2004) may be useful to definitively investigate the fusion of tips. Investigation into the effects of GDNF on organ culture highlights that GDNF acts to increase adhesion between ureteric bud cells and exogenous GDNF can cause fusion of two naked ureteric buds in hanging drop cultures (Sainio *et al.* 1997). It is possible that in order for tips of the ureteric bud to fuse, a threshold concentration of GDNF is needed in the vicinity, but also the tips must be in close association for the cells to intermingle with each other. It was difficult to measure the distances between tips so it is not possible to know whether a threshold distance between tips is always maintained.

The clustered kidneys could provide some insight into the abnormal development of fused kidneys. Fused kidneys have been linked to pathologies such as hypertension and renal calculi formation (Almange *et al.* 1978; Raj *et al.* 2004). It is generally accepted that fused kidneys form due to the induction of extra ureteric buds and more focused investigations have suggested that fused kidneys can form due to abberant migration of nephrogenic mesenchyme (Domenech-Mateu *et al.* 1988) or defects in renal capsule development (Levinson *et al.* 2005). It is unclear from the literature whether the collecting ducts of the fused kidneys anastomose but it is plausible that based on the data from the clustered kidneys that branched collecting duct systems are abnormal. The Foxd1 mutant presents with fused pelvic kidneys which show decreased ureteric bud branching (although surprisingly the number of nephrons is normal) (Levinson *et al.* 2005). It would be interesting to look at the development of the renal capsule (by Foxd1 expression analyses) in the kidney clusters to see how the capsule develops and whether it forms around the periphery of the cluster or around each individual epithelial tree.

5.4.4 A minimum distance between tips

Previous morphometric investigations of kidneys in organ culture assume the kidney is essentially 2 dimensional (Lin *et al.* 2003; Bush *et al.* 2004; Steer *et al.*

2004). Scanning electron micrographs of cultured kidneys suggest that there is little depth to a kidney culture as the tubular epithelia are pronounced on the organ surface and do not appear buried within a great tissue thickness (figure 5.28, from J. Davies).

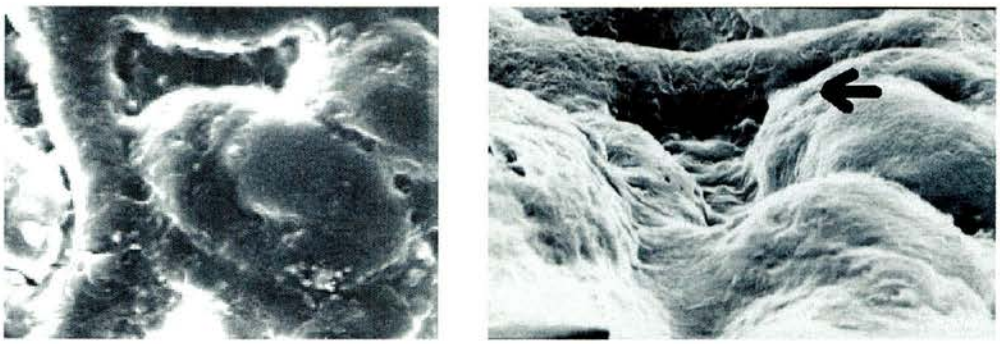


Figure 5.28: SEM of cultured kidneys
The above images from Jamie Davies are SEM images of a cultured kidney. Image size is unknown but is approximately 200μm. These images suggest the kidney is not appreciably thick as the tubules stand proud on the surface of the tissue.

However, it seems from the confocal images of clustered and isolated kidneys that the kidney has an appreciable depth when cultured (*ex vivo*). Smaller optical sections (~0.5μm) would have allowed 3D reconstruction of the kidney cultures although due to the complexity of the kidney shapes I think image processing programs would have to have been employed to help analyse the 3D digital images produced. Others have also recognised the 3D nature of cultured kidneys and have begun to develop image analysis programs to incorporate this (Cullen-McEwen *et al.* 2002). The algorithm designed by Cullen-McEwen *et al.* converts a series of confocal optical sections of a cultured kidney (up to 36hr) into a 3D skeleton which can be used for morphometric analysis. The authors do recognise some limitations of this algorithm including the inability of the program to automatically recognise overlapping branching which become joined during skeletonisation of the images. The authors used anti-calbindin-D_{28k} to stain the ureteric bud and had to manually trace the positions of the weaker staining collecting ducts and they suggest that other methods should be used to uniformly highlight the bud (Cullen-McEwen *et al.* 2002).

Unfortunately this method of analysis was not suitable for larger kidneys (*i.e.* longer than 48hr cultured kidneys) as they were too big for the (512 x 512 pixel) frame of the confocal microscope used and suggested tilling software and a motorised stage would help to image larger kidneys.

Histological sectioning of the kidney cultures is also an option to understand the 3D nature of the clustered and isolated kidneys. Again, image analysis software would have to be employed to carefully reconstruct the ureteric bud pattern. Another imaging technique used to create 3D images of developing organs and embryos is optical projection tomography (OPT) (Sharpe 2003; Sharpe 2004). It has extra advantages over confocal microscopy in that larger specimens such as embryos can be imaged (Sharpe 2003; Sharpe 2004). In my experiments, sometimes the clustered kidneys were slightly thicker than the 160μm limit of the confocal microscope used and so OPT may be useful in overcoming this. OPT is also an attractive option to analyse kidneys in 3D as it can be used for detecting stains other than fluorescent dyes (Sharpe 2003; Sharpe 2004). Therefore OPT would be useful to analyse the positioning of tips within developing kidneys using more specific tip markers (such as *Wnt11)* by *in situ* hybridisation.

If there is a minimum distance between tips observed two possibilities must be explored. Firstly, is this minimum distance between tips independent or dependent on the stage of branching morphogenesis? In other words, do early kidneys observe a greater distance between the tips compared to more developed kidneys? Or do the tips of kidneys observe the same distance between tips at all stages of branching. Secondly, how would the idea of a minimum distance between tips fit in with the process of branching morphogenesis itself? When a tip branches into two daughter branches, the two new tips move in opposite directions and intervening stalk region elongates. It is possible that the movement of sister tips in opposite directions is a reactive measure to establish a minimum tip distance between them. Although it is unclear how the branching event is initiated, I propose that this branching event may only proceed if there is less repulsive signal on the 'outside' of the developing tips relative to an increasingly concentrated signal on the 'cleft' side (figure 5.29). Would this mean that the tips are somewhat mediolaterally polarised with regard to gene expression during branching or is it a passive build up of signals in the cleft due to its

layout? Until tip-tip signalling can be effectively visualised and measured this remains unknown.

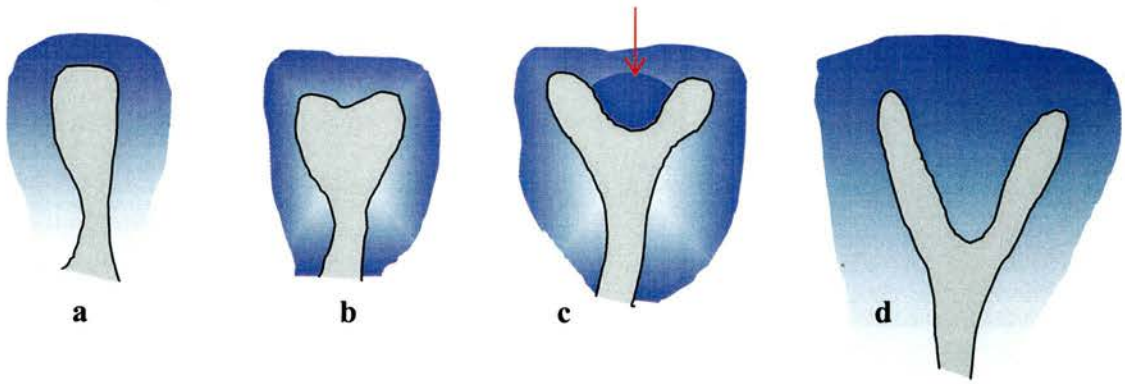


Figure 5.29: Possible mechanism of branching.

It maybe that that spacing of the ureteric bud branches is controlled by maintaining a minimum tip distance between branches. How would the process of branching occur if threshold tip distances were observed? When a minimum tip distance is maintained, it is possible that a tip will not branch (a). It is in equal proximity to tips in all directions and it is sensing equal concentrations of repulsive signal form other tips in all directions (a). If there was a drop in this signal, suggesting that there is 'tip-free' space in which to invade, the tip may be stimulated to undergo branching morphogenesis (b). As a result two sister tips form, each making there own repulsive signal (c). Localised increases in repulsive signal would have to be produced in the branch cleft (red arrow) in order for the tips to react to each other and move away. The tips then moved an appropriate distance and direction away from each other so that they are positioned optimally relative to other tips (d) and are sensing equal concentrations of repulsive signal from the other tips in all directions (compare a and d).

5.4.5 Anti-calbindin-D_{28k} staining

All organ culture experiments outlined in this chapter used anti-calbindin-D_{28k}. This stain was an obvious choice because it does not bind to the developing nephrons, stroma or the nephrogenic mesenchyme. However there were some problems with this stain that were not realised until analysis was underway. Firstly calbindin-D_{28k} expression was turned on in the connecting duct regions of the collecting duct system. Calbindin-D_{28k} has been previously reported to show up in the connecting ducts (Davies 1994) and its expression in this area is dependent on Vitamin D (Davies 1994). As serum supplemented medium was changed daily on the

kidney cultures, levels of Vitamin D were probably maintained possibly leading to the expression of calbindin-D_{28k} in the connecting ducts. Therefore calbindin-D_{28k} stains connecting ducts and the collecting ducts indiscriminately in the cultures. As a result, connecting ducts could have unknowingly been treated as tips and included in the number of tips counted per kidney. This does not present a major problem as the connecting ducts arise from the ureteric bud (McIntosh *et al.* 1986; Howie *et al.* 1993; Liu *et al.* 1993) and so one can argue that they are tips of the ureteric bud. However the overall number of tips per kidney could be significantly affected if the rates of nephrogenesis between kidneys in culture and in cluster are different. If the number of mature nephrons differs between kidneys in cluster and kidneys in culture then there would be a proportionate increase/decrease in the number of connecting ducts which would be reflected in the number of tips per kidney. Staining of the clusters with anti-laminin was attempted but was not very useful as the staining was weaker in the core of the clusters, possibly due to increased thickness of the culture, so quantification of the numbers of developing nephrons was difficult.

Anti-calbindin-D_{28k} staining was often weaker in the centre of the clustered kidney cultures even after adjusting the levels of contrast. This was possibly due to the inability of the antibody to penetrate into the thicker tissue.

Although anti-calbindin-D_{28k} staining of early cultures of kidneys was uniform throughout the ureteric bud, in the 120hr and 168hr cultures there was comparatively weaker staining in the proximal branches relative to the distal branches of the ureteric bud. This decrease in calbindin-D_{28k} protein in the proximal branches of the ureteric bud has been highlighted previously in rabbit embryonic kidneys (McIntosh *et al.* 1986) and mRNA for calbindin-D_{28k} is only present in the cortically collecting ducts (Liu *et al.* 1996). This however limits the usefulness of calbindin-D_{28k} in elucidating the branching patterns because the older branches become more difficult to trace as the kidney matures. Other stains which specifically highlight the entire ureteric bud irrespective of the stage of development would be very useful to overcome these limitations for example using kidneys which express GFP under the hoxb7 promoter (Srinivas *et al.* 1999b).

5.4.6 Centripetal growth

In some kidney cultures (both kidney in cluster and in isolation) the tips of the ureteric bud seemed to be moving centripetally instead of the normal centrifugal movement. There may be many explanations for this. The tips may be joining up with nephrons to form arched connecting tubules or the tips may be attracted to mesenchyme on the outer surface of the kidney which, due to the flattening of the organ in culture, did not get sufficiently induced initially.

The overall aim of these investigations was to determine the reaction of the tips of the ureteric bud when they are forced into close opposition with each other. Principally, development of the ureteric bud was abnormal in two ways. It appeared that the ureteric buds were substantially under branched and in fact branching morphogenesis ceased earlier than normal. In addition to this, the epithelial trees had an abnormal shape and appeared to be compressed in the direction of the collision with the neighbouring clustered kidney. This supports the idea that the tips of the ureteric bud interact with each other to avoid colliding. Also, the relative positioning of tips at the periphery of each kidney with respect to the nearest opposing tips maybe random, as there was not significant evidence to suggest otherwise. Although this specific finding does not suggest that tips interact with each other to influence their positioning, it is possible that other spacing mechanisms are work to ensure tips of the branching ureteric bud avoid colliding with each other.

Chapter 6

Conclusions

The conclusion will concentrate on the following topics:

- Experimental conclusions
- Tip/stalk cell identity
- A role for GDNF in establishing tip/stalk cell populations?
- Guidance mechanisms
- Future investigations
- Summary

6.1 Experimental conclusions

My work sought to investigate a number of avenues relating to the regulation of cell behaviour and identity in the branching epithelium, the ureteric bud. Firstly I characterised the binding pattern of *Dolichos biflorus* agglutinin, a novel marker of stalks of the ureteric bud. DBA respects the same boundary as the tip marker Wnt11 (assuming the techniques of lectin histochemistry and *insitu* hybridisation do not differentially change the overall size of the kidney). Therefore it seems that the ureteric bud strictly regulates the distinction of the cells into stalk and tip cell populations. Using DBA as a marker of the stalks of the ureteric bud along with *Wnt11* as a marker of tip cells, I tested the hypothesis that the tips of the ureteric bud undergo a change in identity and become stalk-like when branching morphogenesis of the ureteric bud is inhibited. The tips of the ureteric bud did indeed undergo this change to a stalk-like identity when kidneys were grown in the branching inhibitor, sodium chlorate. It has been shown before that tips loose the expression of tip markers such as *Wnt11* when inhibited from branching (Kispert *et al.* 1996) but this study is the first to demonstrate that concomitantly the tips convert to stalk-like cells. It would be interesting to investigate whether the change in cell identity also occurs when kidneys are exposed to other exogenous factors implicated in branching morphogenesis.

It seems that the two cell populations of the ureteric bud are different with regard to their behaviour and also their molecular profiles. Yet the two cell types are interchangeable (figure 6.1).

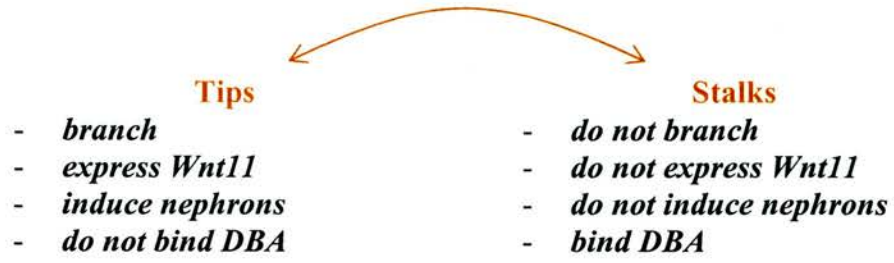


Figure 6.1: Tips and stalks are interchangeable cell types

The plastic nature of the cells of the ureteric bud was further confirmed through a series of experiments in which isolated stalk regions were enticed to form tips. It was proposed that tips produce the majority of the branching events during ureteric bud branching because the stalks of the ureteric bud were differentiated to such an extent to have lost the ability to from branches. Stalk cultures did generate tips and were capable of undergoing multiple rounds of branching, so this hypothesis was rejected. A variation on the stalk cultures was carried out to show that stalk regions can undergo branching even when tips are formed elsewhere in the epithelium. This tends to rule out a mechanism by which tips suppress branch formation from the stalk via long range signals propagated through the epithelium.

The final sets of experiments focused on investigating tip-tip interactions and the role of these interactions in the spacing of branches of the ureteric bud. Kidneys were placed on a collision course with one another and their reaction was determined using a number of methods. When kidneys were cultured in close opposition with each other, they were under branched and more flattened in their shape compared to isolated kidneys. They did not grow as far as expected towards the direction of the kidney with which they are forced to collide. This evidence fits well with a model in which tip-tip interactions, whether direct or indirect via the mesenchyme, influenced the overall shape the ureteric bud. However, it is possible that the affects seen in the clustered cultures are a result of experimental culture conditions *i.e.* restricted diffusion of nutrients or limited space in the core of the clusters could have an adverse affect on the growth of the clustered kidneys. In order to fully understand and account for such experimental affects more insightful comparative controls could

be used such as cultures of single kidneys surrounded by mesenchyme of 3 extra kidneys.

There was not significant evidence to suggest that the tips at the periphery of the kidneys in cluster are non-randomly positioned with respect to the tips of the opposing kidney. This does not support the hypothesis that tips can influence the spacing of surrounding tips although it is possible that another process is needed to implement such a mechanism. Perhaps a critical distance is always maintained between tips so that collisions are avoided and branching continues until this minimum distance is reached. In the clustered kidneys, branching morphogenesis unexpectedly finished by 120hr of culture if not earlier although continued elongation of branches could mean that at later stages of development a new minimum tip distance is tended towards. I was unable, due to the limitations of the imaging methods and analytical tool used, to accurately measure the absolute distances between tips so I could not concluded either way whether a minimum distance is maintained between tips. Neither was I able to ascertain whether the minimum distance (if it exists) varied over time (i.e. the stage of kidney development) or was a constant distance which the tips tended to maintain regardless of the culture length. Overall, this set of experiments represent the first attempts to try and dissect the role tip-tip interactions play in ureteric bud branching. They support and limit the theory that tip-tip interactions are important in controlling kidney development. In future work it would be interesting to apply these investigations to other model systems of branching morphogenesis such as cell and mesenchyme-free culture models. However, in order to apply these studies to other branching models, more suitable methods of imaging and analysing complex 3D structures would have to be found.

6.2 Tip/stalk cell identity

Viscous fingering as a model of branching (Fleury *et al.* 2002) falls short of describing the regular pattern found with branching epithelia. It is possible that the branching of the ureteric bud relies on a combination of random tip splitting in response to mechanical extracellular/mesenchymal pressures (Fleury *et al.* 2002), an

intrinsic branching program of the epithelium itself and a shaping and moulding effect of positive and negative mesenchymal derived growth factors. A period of remodelling of ureteric bud branches follows the period of branching morphogenesis and encompasses such events as rapprochement of the collecting ducts (Oliver 1968). Although it is uncertain whether the tips always keep a 'safe' distance from each other by communicating with each other, it is possible.

Some interesting questions are still proposed, but as of yet unanswered. How is stalk/tip identity established and controlled during kidney development? Is it possible that there are more than two cell population within early ureteric bud? What makes a tip cell change into a stalk cell and what holds the stalk cells in this differentiation state?

My investigations have shown that the tips and stalks appear to respect the same boundary (as evidenced by the fact that the *Wnt11* expression domain is the same distance from the edge of the tip as the beginning of the DBA binding domain is from the edge). This would suggest that there are not more than two cell populations within the ureteric bud, at least at this stage of development. However, as I employed two different methods to measure the length of a tip (DBA lectin histochemistry and *Wnt11 insitu* hybridisation) it must be recognised that these two methods may affect the overall size of the kidney rudiments in different ways.

6.3 A role for GDNF in establishing tip/stalk cell populations?

It has been suggested that tip/stalk identity is controlled by GDNF signalling through its receptor RET. Microarray analysis of ureteric bud tips and stalks has uncovered many differentially expressed genes and it is pointed out that a number genes expressed specifically by the tip are targets of GDNF signalling (B. Lu and F. Costantini, unpublished observation) which suggests that signalling through RET is an important mechanism for specifying tip-stalk identity (Schmidt-Ott *et al.* 2005). It is also suggested that even in 3D culture of isolated ureteric buds that the distinction between tips and stalks is maintained as *Wnt11* is restricted to the tips of the ureteric bud in these cultures (B. Lu and F. Costantini, unpublished observation (Shakya *et al.* 2005b)). GDNF is still thought to be required to induce branching behaviour as

the rescued kidneys of *Gdnf*$^{-/-}$ mice, in which *Gdnf* is expressed ectopically in the collecting duct epithelium (ureteric bud and wolffian duct), are histologically normal (Shakya *et al.* 2005b).

Therefore, it could be predicted that if stalk cells were activated by GDNF they would become tip like and proliferate rapidly. However when *Gdnf* is expressed in the entire collecting duct system under the *Hoxb7* promoter, a number of interesting results are observed. There isn't a uniform swelling of the ducts in these mice. Instead ectopic ureteric bud are formed (Shakya *et al.* 2005b). The cell identity of these buds was normal, with distinct tip cell populations showing specific expression of *Ret*, *Wnt11* and *Gfrα-1* (Shakya *et al.* 2005b). The authors suggest that there is a region of the Wolffian duct that sends out inhibitory signals to repress budding in the adjacent region of the duct (Shakya *et al.* 2005b). The experiments presented in this thesis suggest also that there are inhibitory signals from the tips to the nearby stalks. Although the nature of these signals remains unclear, it appears that these signals do not seem to cause irreversible differentiation of the stalks. There does not seem to be inhibitory signals passing through the epithelial cells themselves to suppress branch formation from the stalks.

Another fact argues against GDNF signalling being the master regulator of tip and stalk cell identity. When exogenous GDNF is added to a culture of kidney where the ureteric bud expresses *Ret* in all its cells, or when constitutively active RET is expressed throughout the ureteric bud, there is no abnormal branching or unusual growth of the ureteric bud stalks (Srinivas *et al.* 1999a). Therefore it is most likely that although GDNF signalling is required for branching the signalling maybe suppressed by the cells of the stalks somewhere downstream in the GDNF signalling cascade. It is also plausible that the tips inhibit stalk branching using shorter range signals that would dissipate as the stalks became further away from the tips (and it should be considered that the range of these signals may not have been effective in the culture set ups I carried out).

Chimeric ureteric buds which express a mixture of *Ret*$^{-/-}$ cells and wildtype *Ret* cells show that the tips of the ureteric bud tend to exclude the mutant cells (Shakya *et al.* 2005a), possibly because they are not capable of thriving in a microenviroment

where GDNF is a principle growth factor. Instead they thrive in a niche which depends on the 'stalk' growth factor.

This evidence would argue against a model where localised GDNF establishes tip and stalk cells and suggests that proximo-distal patterning of the ureteric bud is intrinsic to the epithelium itself (Shakya *et al.* 2005b). The role of GDNF in establishing tip and stalk cell identity is unclear (Shakya *et al.* 2005b). It is essential for ureteric bud branching but its spatial localisation does not appear to be important. It is possibly the case that tip identity is, while stalk identity is not, dependent on GDNF signalling.

6.4 Guidance mechanisms

Few potential guidance mechanisms intrinsic to the ureteric bud have been suggested. One suggested candidate system are the ephs and ephrins (Miao *et al.* 2003) which have been shown in cell culture models to have a role in controlling branching morphogenesis (Miao *et al.* 2003). Little is known about the role of ephs and ephrins in metanephric development although at least one receptor *Epha2* is expressed by the cells of the ureteric bud (Miao *et al.* 2003). As the eph and ephrin receptors require cell-to-cell contact for signalling (Wilkinson 2001) and the ureteric bud tips do not appear to contact each other, it is more plausible that the mechanisms controlling branching are modulated between branches and mesenchyme or that the signalling between tips is mediated by a secreted signal. In accordance with this, the tips of the ureteric bud have been shown to be abundant in endoplasmic reticulum (Meyer *et al.* 2004) which is associated with high rates of protein synthesis. The idea that mesenchyme regulates the spacing out of branches is an attractive one and the mesenchymally derived molecule, TGFβ1, has already been shown to specifically affect the angles formed between branches during kidney development (Bush *et al.* 2004). It may be the case that tips will only move into a compartment of the mesenchyme if there is enough virgin mesenchyme to support their growth. The kidney grows roughly exponentially (Foley *et al.* 2002; Davies *et al.* 1998) and the growth of the tips at the periphery of the metanephros may be driven by exponential expansion of the surface area. The tips are always moving into a rapidly expansive

space and the rate at which the boarder rim of mesenchyme can expand most likely effects the rate of tip branching. Therefore, it may be that a threshold distance between tips is maintained not as a result of tip to tip interactions but because levels of virgin mesenchyme in the vicinity are not critical enough to entice the tip to grow or branch further.

6.5 Future investigations

Future investigations should focus on how tip and stalk cell identity is established in the ureteric bud epithelium. The ability to culture ureteric buds independently of mesenchyme may be very useful in investigating this phenomenon. The investigation of the initial outgrowth of the ureteric bud (at E10.5) would be an interesting situation to study how tips and stalks become specified as it is at this stage that the distinction of these cell populations first occurs. Also useful would be the use of hanging drop cultures of ureteric buds. In hanging drop cultures the ureteric bud rounds up so that it looses its morphological distinction into tips and stalks (Sainio *et al.* 1997). However it is unclear how cell identity is modulated in this situation and how, if possible, distinct populations of tip and stalk cells re-establish themselves when, for example, a ureteric bud is cultured for sometime in a hanging drop and is then enticed to branch in a 3D matrix. It would also be interesting to carry out microarray analysis to identify cell markers of 3D cultures of mesenchyme free ureteric buds. Immunohistochemical/*insitu* hybridisation would also be needed to verify the localisation/expression pattern of putative markers suggested from the array data. These types of analyses would shed light on the inherent ability of a bud to regulate its cell identity and hopefully add to our understanding of ureteric bud intrinsic branching mechanisms. Limitations to naked ureteric bud microarray analyses may include the technical difficulty in separating the ureteric bud into two pure populations of tip and stalk cells.

Another avenue for future investigation is the specific role of stalks of the ureteric bud in kidney development. The maturation of these regions of the ureteric bud may be regulated by an intrinsic program of differentiation but the stalks may also function as modulators for a number of developmental processes including the

maturation of the medullary interstitium, nephron maturation (Loop of Henle), or even the development of other tissue components vital for renal organogenesis such as vasculogenesis and innervation (Schmidt-Ott *et al.* 2005).

6.6 Summary

Overall the distinction between tips and stalk cells of the ureteric bud is important for ensuring branching morphogenesis proceeds in a controlled manner. Based on my experiments presented in this thesis, I have concluded that the distinction between stalks and tips can be disrupted when branching is inhibited and that the differentiation of tips into stalks is not an irreversible one. The shaping and directed growth of the ureteric bud may be influenced by other ureteric buds which are cultured nearby suggesting there may be interactions between tips (although more detailed studies are needed to investigate this further). It is critical that the underlying molecular mechanisms of tip-stalk cell distinction are investigated if we are to fully understand the process of ureteric bud branching morphogenesis.

Chapter 7

References

Reference List

Abbate M, Brown D, Bonventre JV (1999) Expression of NCAM recapitulates tubulogenic development in kidneys recovering from acute ischemia. *AJP - Renal Physiol* **277,** F454-F463.

Abdelhak S, Kalatzis V, Heilig R, Compain S, Samson D, Vincent C, Levi-Acobas F, Cruaud C, Le Merrer M, Mathieu M, Konig R, Vigneron J, Weissenbach J, Petit C, Weil D (1997) Clustering of mutations responsible for branchio-oto-renal (BOR) syndrome in the eyes absent homologous region (eyaHR) of EYA1. *Hum Mole Genetics* **6,** 2247-2255.

Airalsinen MS, Saarma M (2002) The GDNF family: Signalling, biological functions and therapeutic value. *Nat Rev Neurosci* **3,** 383-394.

al-Awqati Q, Goldberg MR (1998) Architectural patterns in branching morphogenesis in the kidney. *Kidney Int* **54,** 1832-1842.

al-Awqati Q, Vijayakumar S, Takito J, Hikita C, Yan L, Wiederholt T (2000) Phenotypic plasticity and terminal differentiation of the intercalated cell: the hensin pathway. *Exp Nephro* **8,** 66-71.

al-Awqati Q, Oliver JA (2002) Stem cells in the kidney. *Kidney Int* **61**, 387-395.

al-Awqati Q, Schwartz GJ (2004) A fork in the road of cell differentiation in the kidney tubule. *J Clin Invest* **113,** 1528-1530.

Almange C, Guivarch G, Colin G, Lafitte G, Leborgne P (1978) Horseshoe kidney and hypertension (author's transl). *Sem Hop* **54,** 1442-1444.

Anderson RJ, Ray CJ, Popoff MR (2000) Evidence for Rho protein regulation of renal tubular epithelial cell function. *Kidney Int* **58,** 1996-2006.

Anglani F, Forino M, Del Prete D, Tosetto E, Torregrossa R, D'Angelo A (2004) In search of adult renal stem cells. *J Cell Mol Med* **8,** 474-487.

Bao Q, Hughes RC (1999) Galectin-3 and polarized growth within collagen gels of wild-type and ricin-resistant MDCK renal epithelial cells. *Glycobiology* **9,** 489-495.

Barasch J, Yang J, Qiao J, Tempst P, Erdjument-Bromage H, Leung W, Oliver JA (1999) Tissue inhibitor of metalloproteinase-2 stimulates mesenchymal growth and regulates epithelial branching during morphogenesis of the rat metanephros. *J Clin Invest* **103,** 1299-1307.

Barasch J, Pressler L, Connor J, Malik A (1996) A ureteric bud cell line induces nephrogenesis in two steps by two distinct signals. *AJP - Renal Physiol* **271,** F50-F61.

Barkhordari A, Stoddart RW, McClure SF, McClure J (2004) Lectin histochemistry of normal human lung. *J Mol Histol* **35,** 147-156.

Barnett MW, Fisher CE, Perona-Wright G, Davies JA (2002) Signalling by glial cell line-derived neurotrophic factor (GDNF) requires heparan sulphate glycosaminoglycan. *J Cell Sci* **115,** 4495-4503.

Beitel GJ, Krasnow MA (2000) Genetic control of epithelial tube size in the Drosophila tracheal system. *Development* **127,** 3271-3282.

Beuerman RW, Thompson HW (1992) Molecular and cellular responses of the corneal epithelium to wound healing. *Acta Ophthalmol Suppl* **202,** 7-12.

Bladt F, Riethmacher D, Isenmann S, Aguzzi A, Birchmeier C (1995) Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* **376,** 768-771.

Boivin GP, O'Toole BA, Orsmby IE, Diebold RJ, Eis MJ, Doetschman T, Kier AB (1995) Onset and progression of pathological lesions in transforming growth factor-beta 1-deficient mice. *Am J Pathol* **146,** 276-288.

Bonventre JV (2003) Dedifferentiation and Proliferation of Surviving Epithelial Cells in Acute Renal Failure. *J Am Soc Nephrol* **14,** S55-S61.

Boyden EA (1927) Experimental obstruction of the mesonephric ducts. *Proc Soc Exp Biol Med* **24,** 572-576.

Bramwell VH, Crowther D, Gallagher J, Stoddart RW (1982) Studies of lectin binding to normal and neoplastic lymphoid tissues. I. Normal nodes and Hodgkin's disease. *Br J Cancer* **46,** 568-581.

Brinck U, Bosbach R, Korabiowska M, Schauer A, Gabius HJ (1995) Lectin-binding sites in the epithelium of normal human appendix vermiformis and in acute appendicitis. *Histol Histopathol* **10,** 61-70.

Brodbeck S, Besenbeck B, Englert C (2004) The transcription factor Six2 activates expression of the Gdnf gene as well as its own promoter. *Mech Dev* **121,** 1211-1222.

Brophy PD, Ostrom L, Lang KM, Dressler GR (2001) Regulation of ureteric bud outgrowth by Pax2-dependent activation of the glial derived neurotrophic factor gene. *Development* **128,** 4747-4756.

Brown G, Hughes PJ, Michell RH (2003) Cell differentiation and proliferation--simultaneous but independent? *Exp Cell Res* **291,** 282-288.

Bullock SL, Fletcher JM, Beddington RS, Wilson VA (1998) Renal agenesis in mice homozygous for a gene trap mutation in the gene encoding heparan sulfate 2-sulfotransferase. *Genes Dev* **12,** 1894-1906.

Bush KT, Sakurai H, Steer DL, Leonard MO, Sampogna RV, Meyer TN, Schwesinger C, Qiao J, Nigam SK (2004) TGFβ superfamily members modulate growth, branching, shaping, and patterning of the ureteric bud. *Dev Biol* **266,** 285-298.

Cancilla B, Ford-Perriss MD, Bertram JF (1999) Expression and localization of fibroblast growth factors and fibroblast growth factor receptors in the developing rat kidney. *Kidney Int* **56,** 2025-2039.

Cantley LG, Barros EJ, Gandhi M, Rauchman M, Nigam SK (1994) Regulation of mitogenesis, motogenesis, and tubulogenesis by hepatocyte growth factor in renal collecting duct cells. *Am J Physiol* **267,** F271-F280.

Carroll TJ, Park JS, Hayashi S, Majumdar A, McMahon AP (2005) Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the Mammalian urogenital system. *Dev Cell* **9,** 283-292.

Cebrián C, Borodo K, Charles N, Herzlinger DA (2004) Morphometric index of the developing murine kidney. *Dev Dyn* **231,** 601-608.

Celli G, LaRochelle WJ, Mackem S, Sharp R, Merlino G (1998) Soluble dominant-negative receptor uncovers essential roles for fibroblast growth factors in multi-organ induction and patterning. *EMBO J* **17,** 1642-1655.

Chen D, Roberts R, Pohl M, Nigam S, Kreidberg J, Wang Z, Heino J, Ivaska J, Coffa S, Harris RC, Pozzi A, Zent R (2004) Differential expression of collagen- and laminin-binding integrins mediates ureteric bud and inner medullary collecting duct cell tubulogenesis. *Am J Physiol Renal Physiol* **287,** F602-F611.

Christiansen JH, Dennis CL, Wicking CA, Monkley SJ, Wilkinson DG, Wainwright BJ (1995) Murine Wnt-11 and Wnt-12 have temporally and spatially restricted expression patterns during embryonic development. *Mech Dev* **51,** 341-350.

Christiansen JH, Monkley SJ, Wainwright BJ (1996) Murine WNT11 is a secreted glycoprotein that morphologically transforms mammary epithelial cells. *Oncogene* **12,** 2705-2711.

Clark AT, Bertram JF (1999) Molecular regulation of nephron endowment. *AJP - Renal Phys* **276,** F485-F497.

Cullen-McEwen LA, Fricout G, Harper IS, Jeulin D, Bertram JF (2002) Quantitation of 3D ureteric branching morphogenesis in cultured embryonic mouse kidney. *Int J Dev Biol* **46,** 1049-1055.

Cullen-McEwen LA, Kett MM, Dowling J, Anderson WP, Bertram JF (2003) Nephron number, renal function, and arterial pressure in aged GDNF heterozygous mice. *Hypertension* **41,** 335-340.

Davies J (1994) Control of calbindin-D28K expression in developing mouse kidney. *Dev Dyn* **199,** 45-51.

Davies J, Lyon M, Gallagher J, Garrod D (1995) Sulphated proteoglycan is required for collecting duct growth and branching but not nephron formation during kidney development. *Development* **121,** 1507-1517.

Davies JA, Bard JB (1998) The development of the kidney. *Curr Top Dev Biol* **39,** 245-301.

Davies JA, Millar CB, Johnson EM, Jr., Milbrandt J (1999) Neurturin: an autocrine regulator of renal collecting duct development. *Dev Genet* **24,** 284-292.

Davies JA, Fisher CE, Barnett MW (2001) Glycosaminoglycans in the study of mammalian organ development. *Biochem Soc Trans* **29,** 166-171.

Davies JA, Fisher CE (2002) Genes and proteins in renal development. *Exp Nephrol* **10,** 102-113.

Davies JA (2002) Morphogenesis of the metanephric kidney. *Scientific World Journal* **2,** 1937-1950.

Davies JA, Yates EA, Turnbull JE (2003) Structural determinants of heparan sulphate modulation of GDNF signalling. *Growth Factors* **21,** 109-119.

Davies JA (2005) The Kidney Development Database. *http://golgi.ana.ed.ac.uk/kidhome.html.*

De Calisto J, Araya C, Marchant L, Riaz CF, Mayor R (2005) Essential role of non-canonical Wnt signalling in neural crest migration. *Development* **132,** 2587-2597.

de Graaff E, Srinivas S, Kilkenny C, D'Agati V, Mankoo B, Costantini F, Pachnis V (2001) Differential activities of the RET tyrosine kinase receptor isoforms during mammalian embryogenesis. *Genes Dev* **15,** 2433-2444.

Desai LP, Aryal AM, Ceacareanu B, Hassid A, Waters CM (2004) RhoA and Rac1 are both required for efficient wound closure of airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **287,** L1134-L1144.

Devarajan P, Mishra J, Supavekin S, Patterson LT, Steven PS (2003) Gene expression in early ischemic renal injury: clues towards pathogenesis, biomarker discovery, and novel therapeutics. *Mol Genet Metab* **80,** 365-376.

Devuyst O, Burrow CR, Smith BL, Agre P, Knepper MA, Wilson PD (1996) Expression of aquaporins-1 and -2 during nephrogenesis and in autosomal dominant polycystic kidney disease. *Am J Physiol* **271,** F169-F183.

Dickinson H, Walker DW, Cullen-McEwen L, Wintour EM, Moritz K (2005) The spiny mouse (Acomys cahirinus) completes nephrogenesis before birth. *AJP - Renal Physiol* **289,** F273-F279.

Domenech-Mateu JM, Gonzalez-Compta X (1988) Horseshoe kidney: a new theory on its embryogenesis based on the study of a 16-mm human embryo. *Anat Rec* **222,** 408-417.

Donovan MJ, Natoli TA, Sainio K, Amstutz A, Jaenisch R, Sariola H, Kreidberg JA (1999) Initial differentiation of the metanephric mesenchyme is independent of WT1 and the ureteric bud. *Dev Genet* **24,** 252-262.

Dressler G (2002) Tubulogenesis in the developing mammalian kidney. *Trends Cell Biol* **12,** 390-395.

Dudley AT, Lyons KM, Robertson EJ (1995) A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev* **9,** 2795-2807.

Dudley AT, Godin RE, Robertson EJ (1999) Interaction between FGF and BMP signalling pathways regulates development of metanephric mesenchyme. *Genes Dev* **13,** 1601-1613.

Dvorankova B, Motlik J, Holikova Z, Vacik J, Smetana K, Jr. (2002) Dolichos biflorus agglutinin-binding site expression in basal keratinocytes is associated with cell differentiation. *Biol Cell* **94,** 365-373.

Dziarmaga A, Clark P, Stayner C, Julien JP, Torban E, Goodyer P, Eccles M (2003) Ureteric Bud Apoptosis and Renal Hypoplasia in Transgenic PAX2-Bax Fetal Mice Mimics the Renal-Coloboma Syndrome. *J Am Soc Nephrol* **14,** 2767-2774.

Eisner BH, Bloom DA (2002) Wolff and Muller: fundamental eponyms of embryology, nephrology and urology. *J Urol* **168,** 425-428.

Engel U, Breborowicz D, Bog-Hansen T, Francis D (1997) Lectin staining of renal tubules in normal kidney. *APMIS* **105,** 31-34.

Enomoto H, Araki T, Jackman A, Heuckeroth RO, Snider WD, Johnson EM, Jr., Milbrandt J (1998) GFR alpha1-deficient mice have deficits in the enteric nervous system and kidneys. *Neuron* **21,** 317-324.

Etzler ME, Kabat EA (1970) Purification and characterization of a lectin (plant hemagglutinin) with blood group A specificity from Dolichos biflorus. *Biochemistry* **9,** 869-877.

Fan QW, Kadomatsu K, Uchimura K, Muramatsu T (1998) Embigin/basigin subgroup of the immunoglobulin superfamily: different modes of expression during mouse embryogenesis and correlated expression with carbohydrate antigenic markers. *Dev Growth Differ* **40,** 277-286.

Favor J, Sandulache R, NeuhauserKlaus A, Pretsch W, Chatterjee B, Senft E, Wurst W, Blanquet V, Grimes P, Sporle R, Schughart K (1996) The mouse Pax2(1Neu) mutation is identical to a human PAX2 mutation in a family with renal-coloboma syndrome and results in developmental defects of the brain, ear, eye, and kidney. *Proc Natl Acad Sci U S A* **93,** 13870-13875.

Feifel E, Krall M, Geibel JP, Pfaller W (1997) Differential activities of H+ extrusion systems in MDCK cells due to extracellular osmolality and pH. *Am J Physiol* **273,** F499-F506.

Fejes-Toth G, Naray-Fejes-Toth A (1993) Differentiation of intercalated cells in culture. *Pediatr Nephrol* **7,** 780-784.

Fisher CE, Michael L, Barnett MW, Davies JA (2001) Erk MAP kinase regulates branching morphogenesis in the developing mouse kidney. *Development* **128,** 4329-4338.

Fleury V, Watanabe T (2002) Morphogenesis of fingers and branched organs: how collagen and fibroblasts break the symmetry of growing biological tissue. *C R Biol* **325,** 571-583.

Foley JGD, Bard JBL (2002) Apoptosis in the cortex of the developing mouse kidney. *J Anat* **201,** 477-484.

Fujii T, Pichel JG, Taira M, Toyama R, Dawid IB, Westphal H (1994) Expression patterns of the murine LIM class homeobox gene lim1 in the developing brain and excretory system. *Dev Dyn* **199,** 73-83.

Fukata M, Nakagawa M, Kaibuchi K (2003) Roles of Rho-family GTPases in cell polarisation and directional migration. *Curr Opin Cell Biol* **15,** 590-597.

Gilbert P, Minuth WW, Bachmann S (1987) Microheterogeneity of the collecting duct system in rabbit kidney as revealed by monoclonal antibodies. *Cell Tissue Res* **248,** 611-618.

Gilbert T (2002) Vitamin A and kidney development. *Nephrology Dialysis Transplantation* **17 Suppl 9,** 78-80.

Godin RE, Robertson EJ, Dudley AT (1999) Role of BMP family members during kidney development. *Int J Dev Biol* **43,** 405-411.

Goldin GV, Hindman HM, Wessells NK (1984) The role of cell proliferation and cellular shape change in branching morphogenesis of the embryonic mouse lung: analysis using aphidicolin and cytochalasins. *J Exp Zool* **232,** 287-296.

Gosche JR, Islam S, Boulanger SC (2005) Congenital diaphragmatic hernia: searching for answers. *Am J Surg* **190,** 324-332.

Grieshammer U, Le M, Plump AS, Wang F, Tessier-Lavigne M, Martin GR (2004) SLIT2-mediated ROBO2 signalling restricts kidney induction to a single site. *Dev Cell* **6,** 709-717.

Grieshammer U, Cebrian C, Ilagan R, Meyers E, Herzlinger D, Martin GR (2005) FGF8 is required for cell survival at distinct stages of nephrogenesis and for regulation of gene expression in nascent nephrons. *Development* **132,** 3847-3857.

Grobstein C (1953) Morphogenetic interaction between embryonic mouse tissues separated by a membrane filter. *Nature* **172,** 869-870.

Grobstein C (1955) Inductive interaction in the development of the mouse metanephros. *J Exp Zool* **130,** 319-340-

Grobstein C (1956) Trans-filter induction of tubules in mouse metanephrogenic mesenchyme. *Exp Cell Res* **10,** 424-440.

Grobstein C (1957) Some transmission characteristics of the tubule-inducing influence on mouse metanephrogenic mesenchyme. *Exp Cell Res* **13,** 575-587.

Grobstein C (1967) Mechanisms of organogenetic tissue interaction. *Natl Cancer Inst Monogr* **26,** 279-299.

Gruenwald P (1937) Zur Entwicklungsmechanik der Urogebital-systems beim Huhn. *Wilhem Roux Arch Entw Mech* **136,** 786-813.

Guenette RS, Sridhar S, Herley M, Mooibroek M, Wong P, Tenniswood M (1997) Embigin, a developmentally expressed member of the immunoglobulin super family, is also expressed during regression of prostate and mammary gland. *Dev Genet* **21,** 268-278.

Gupta IR, Piscione TD, Grisaru S, Phan T, Macias-Silva M, Zhou X, Whiteside C, Wrana JL, Rosenblum ND (1999) Protein Kinase A Is a Negative Regulator of Renal Branching Morphogenesis and Modulates Inhibitory and Stimulatory Bone Morphogenetic Proteins. *J Biol Chem* **274,** 26305-26314.

Hamelryck TW, Loris R, Bouckaert J, Dao-Thi MH, Strecker G, Imberty A, Fernandez E, Wyns L, Etzler ME (1999) Carbohydrate binding, quaternary structure and a

novel hydrophobic binding site in two legume lectin oligomers from Dolichos biflorus. *J Mol Biol* **286,** 1161-1177.

Hammarstrom S, Murphy LA, Goldstein IJ, Etzler ME (1977) Carbohydrate binding specificity of four N-acetyl-D-galactosamine- "specific" lectins: Helix pomatia A hemagglutinin, soy bean agglutinin, lima bean lectin, and Dolichos biflorus lectin. *Biochemistry* **16,** 2750-2755.

Hellmich HL, Kos L, Cho ES, Mahon KA, Zimmer A (1996) Embryonic expression of glial cell-line derived neurotrophic factor (GDNF) suggests multiple developmental roles in neural differentiation and epithelial-mesenchymal interactions. *Mech Dev* **54,** 95-105.

Herzlinger D, Qiao J, Cohen D, Ramakrishna N, Brown AM (1994) Induction of kidney epithelial morphogenesis by cells expressing Wnt-1. *Dev Biol* **166,** 815-818.

Heuckeroth RO, Enomoto H, Grider JR, Golden JP, Hanke JA, Jackman A, Molliver DC, Bardgett ME, Snider WD, Johnson EM, Jr., Milbrandt J (1999) Gene targeting reveals a critical role for neurturin in the development and maintenance of enteric, sensory, and parasympathetic neurons. *Neuron* **22,** 253-263.

Holthofer H, Schulte BA, Spicer SS (1987) Expression of binding sites for Dolichos biflorus agglutinin at the apical aspect of collecting duct cells in rat kidney. *Cell Tissue Res* **249,** 481-485.

Holthofer H, Schulte BA, Spicer SS (1988) Heterogeneity of apical glycoconjugates in kidney collecting ducts: further studies using simultaneous detection of lectin binding sites and immunocytochemical detection of key transport enzymes. *Histochem J* **20,** 471-477.

Holthofer H (1988) Cell type-specific glycoconjugates of collecting duct cells during maturation of the rat kidney. *Cell Tissue Res* **253,** 305-309.

Holthofer H, Kumpulainen T, Rapola J (1990) Polycystic disease of the kidney. Evaluation and classification based on nephron segment and cell-type specific markers. *Lab Invest* **62,** 363-369.

Howie AJ, Smithson N, Rollason TP (1993) Reconsideration of the development of the distal tubule of the human kidney. *J Anat* **183** (Pt 1), 141-147.

Hrdlickova-Cela E, Plzak J, Holikova Z, Dvorankova B, Smetana K, Jr. (2001) Postmitotic basal cells in squamous cell epithelia are identified with Dolichos biflorus agglutinin--functional consequences. *APMIS* **109,** 714-720.

Huber SM, Horster MF (1996) Ontogeny of apical membrane ion conductances and channel expression in cortical collecting duct cells. *Am J Physiol* **271,** F698-F708.

Huber SM, Braun GS, Horster MF (1999) Expression of the epithelial sodium channel (ENaC) during ontogenic differentiation of the renal cortical collecting duct epithelium. *Pflugers Arch* **437,** 491-497.

Hynes PJ, Fraher JP (2004) The development of the male genitourinary system: III. The formation of the spongiose and glandar urethra. *Br J Plastic Surgery* **57,** 203-214.

Itaranta P, Lin Y, Perasaari J, Roel G, Destree O, Vainio S (2002) Wnt-6 is expressed in the ureter bud and induces kidney tubule development in vitro. *Genesis* **32,** 259-268.

Jing S, Wen D, Yu Y, Holst PL, Luo Y, Fang M, Tamir R, Antonio L, Hu Z, Cupples R, Louis JC, Hu S, Altrock BW, Fox GM (1996) GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. *Cell* **85,** 1113-1124.

Joraku A, Machiguchi T, Atala A, Yoo JJ (2005) Organized Kidney Tissue Structures for the Treatment of End Stage Renal Disease. *The Regenerate International Conference and Exposition http://www.regenerate-online.com/abstract_Joraku2.html*

Karihaloo A, Karumanchi SA, Barasch J, Jha V, Nickel CH, Yang J, Grisaru S, Bush KT, Nigam S, Rosenblum ND, Sukhatme VP, Cantley LG (2001) Endostatin regulates branching morphogenesis of renal epithelial cells and ureteric bud. *Proc Natl Acad Sci U S A* **98,** 12509-12514.

Kispert A, Vainio S, Shen L, Rowitch DH, McMahon AP (1996) Proteoglycans are required for maintenance of Wnt-11 expression in the ureter tips. *Development* **122,** 3627-3637.

Kispert A, Vainio S, McMahon AP (1998) Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney. *Development* **125,** 4225-4234.

Kloth S, Gmeiner T, Aigner J, Jennings ML, Rockl W, Minuth WW (1998) Transitional stages in the development of the rabbit renal collecting duct. *Differentiation* **63,** 21-32.

Kobayashi A, Kwan KM, Carroll TJ, McMahon AP, Mendelsohn CL, Behringer RR (2005) Distinct and sequential tissue-specific activities of the LIM-class homeobox gene Lim1 for tubular morphogenesis during kidney development. *Development* **132,** 2809-2823.

Koseki C, Herzlinger D, al Awqati Q (1992) Apoptosis in metanephric development. *J Cell Biol* **119,** 1327-1333.

Kovacs J, Zilahy M, Gomba S (1997) Morphology of cystic renal lesions. Lectin and immuno-histochemical study. *Acta Chir Hung* **36,** 176-178.

Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R (1993) WT-1 is required for early kidney development. *Cell* **74,** 679-691.

Laitinen L, Virtanen I, Saxen L (1987) Changes in the glycosylation pattern during embryonic development of mouse kidney as revealed with lectin conjugates. *J Histochem Cytochem* **35,** 55-65.

Legallicier B, Trugnan G, Murphy G, Lelongt B, Ronco P (2001) Expression of the Type IV Collagenase System during Mouse Kidney Development and Tubule Segmentation. *J Am Soc Nephrol* **12,** 2358-2369.

Lelievre-Pegorier M, Vilar J, Ferrier ML, Moreau E, Freund N, Gilbert T, Merlet-Benichou C (1998) Mild vitamin A deficiency leads to inborn nephron deficit in the rat. *Kidney Int* **54,** 1455-1462.

Leustek T, Saito K (1999) Sulfate Transport and Assimilation in Plants. *Plant Physiol* **120,** 637-644.

Levinson RS, Batourina E, Choi C, Vorontchikhina M, Kitajewski J, Mendelsohn CL (2005) Foxd1-dependent signals control cellularity in the renal capsule, a structure required for normal renal development. *Development* **132,** 529-539.

Lin Y, Zhang S, Rehn M, Itaranta P, Tuukkanen J, Heljasvaara R, Peltoketo H, Pihlajaniemi T, Vainio S (2001) Induced repatterning of type XVIII collagen expression in ureter bud from kidney to lung type: association with sonic hedgehog and ectopic surfactant protein C. *Development* **128,** 1573-1585.

Lin Y, Zhang S, Tuukkanen J, Peltoketo H, Pihlajaniemi T, Vainio S (2003) Patterning parameters associated with the branching of the ureteric bud regulated by epithelial-mesenchymal interactions. *Int J Dev Biol* **47,** 3-13.

Liu H, Wintour EM (2005) Aquaporins in development -- a review. *Reprod Biol Endocrinol* **3,** 18-

Liu L, Dunn ST, Christakos S, Hanson-Painton O, Bourdeau JE (1993) Calbindin-D28k gene expression in the developing mouse kidney. *Kidney Int* **44,** 322-330.

Liu L, Khastgir A, McCauley JM, Dunn ST, Morrissey JH, Christakos S, Hughes MR, Bourdeau JE (1996) RT-PCR microlocalization of mRNAs for calbindin-D28k and vitamin D receptor in the murine nephron. *Am J Physiol* **270,** F677-F681.

Liu Y (2002) Hepatocyte growth factor and the kidney. *Curr Opin Nephrol Hypertens* **11,** 23-30.

Lu J, Qian J, Izvolsky KI, Cardoso WV (2004) Global analysis of genes differentially expressed in branching and non-branching regions of the mouse embryonic lung. *Dev Biol* **273,** 418-435.

Maeshima A, Nojima Y, Kojima I (2001) The role of the activin-follistatin system in the developmental and regeneration processes of the kidney. *Cytokine Growth Factor Rev* **12,** 289-298.

Majumdar A, Vainio S, Kispert A, McMahon J, McMahon AP (2003) Wnt11 and Ret/Gdnf pathways cooperate in regulating ureteric branching during metanephric kidney development. *Development* **130,** 3175-3185.

McIntosh JE, Bourdeau JE, Taylor AN (1986) Immunohistochemical localization of calbindin-D28k during the development of the rabbit nephron. *Anat Rec* **215,** 383-389.

Mendelsohn C, Lohnes D, Decimo D, Lufkin T, LeMeur M, Chambon P, Mark M (1994) Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* **120,** 2749-2771.

Mendelsohn C, Batourina E, Fung S, Gilbert T, Dodd J (1999) Stromal cells mediate retinoid-dependent functions essential for renal development. *Development* **126,** 1139-1148.

Meyer TN, Schwesinger C, Bush KT, Stuart RO, Rose DW, Shah MM, Vaughn DA, Steer DL, Nigam SK (2004) Spatiotemporal regulation of morphogenetic molecules during in vitro branching of the isolated ureteric bud: toward a model of branching through budding in the developing kidney. *Dev Biol* **275,** 44-67.

Miao H, Nickel CH, Cantley LG, Bruggeman LA, Bennardo LN, Wang B (2003) EphA kinase activation regulates HGF-induced epithelial branching morphogenesis. *J Cell Biol* **162,** 1281-1292.

Michael L (2003) Morphogenetic specializations at the tips of the branching epithelium of the kidney. Phd Thesis.

Michael L, Davies JA (2004) Pattern and regulation of cell proliferation during murine ureteric bud development. *J Anat* **204,** 241-255.

Michael L, Sweeney DE, Davies JA (2005) A role for microfilament-based contraction in branching morphogenesis of the ureteric bud. *Kidney Int* **68,** 2010-2018.

Milbrandt J, de Sauvage FJ, Fahrner TJ, Baloh RH, Leitner ML, Tansey MG, Lampe PA, Heuckeroth RO, Kotzbauer PT, Simburger KS, Golden JP, Davies JA, Vejsada R, Kato AC, Hynes M, Sherman D, Nishimura M, Wang LC, Vandlen R, Moffat B, Klein RD, Poulsen K, Gray C, Garces A, Johnson EM, Jr., . (1998) Persephin, a novel neurotrophic factor related to GDNF and neurturin. *Neuron* **20,** 245-253.

Minuth WW, Gilbert P, Rudolph U, Spielman WS (1989) Successive histochemical differentiation steps during postnatal development of the collecting duct in rabbit kidney. *Histochemistry* **93,** 19-25.

Mitchell BS, Schumacher U (1999) The use of the lectin Helix pomatia agglutinin (HPA) as a prognostic indicator and as a tool in cancer research. *Histol.Histopathol* **14,** 217-226.

Miyazaki Y, Oshima K, Fogo A, Hogan BL, Ichikawa I (2000) Bone morphogenetic protein 4 regulates the budding site and elongation of the mouse ureter. *J Clin Invest* **105,** 863-873.

Moll I, Houdek P, Schmidt H, Moll R (1998) Characterization of epidermal wound healing in a human skin organ culture model: acceleration by transplanted keratinocytes. *J Invest Dermatol* **111,** 251-258.

Montesano R, Matsumoto K, Nakamura T, Orci L (1991) Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* **67,** 901-908.

Moore KA, Polte T, Huang S, Shi B, Alsberg E, Sunday ME, Ingber DE (2005) Control of basement membrane remodeling and epithelial branching morphogenesis in embryonic lung by Rho and cytoskeletal tension. *Dev Dyn* **232,** 268-281.

Moore KL, Persaud TV (1998) *The Developing Human, clinically oriented embryology.* W.B. Saunders Company.

Muramatsu T (1993) Midkine (MK), the product of a retinoic acid responsive gene, and pleiotrophin constitute a new protein family regulating growth and differentiation. *Int J Dev Biol* **37,** 183-188.

Nakagawa T, Kitami Y, Watanabe K, Hanai T, Usuda N, Ogawa A (1996) Increase in lectin binding sites on epithelial cells by chronic bladder infection in rats. *Urol Int* **56,** 90-95.

Nakamura E, Kadomatsu K, Yuasa S, Muramatsu H, Mamiya T, Nabeshima T, Fan QW, Ishiguro K, Igakura T, Matsubara S, Kaname T, Horiba M, Saito H, Muramatsu T (1998) Disruption of the midkine gene (Mdk) resulted in altered expression of a calcium binding protein in the hippocampus of infant mice and their abnormal behaviour. *Genes to Cells* **3,** 811-812.

Nigam SK (2003) From the ureteric bud to the penome. *Kidney Int* **64,** 2320-2322.

Nobes CD (2000) Rho GTPases and cell migration-fibroblast wound healing. *Methods Enzymol* **325,** 441-449.

Oliver J (1968) *Nephrons and Kidneys: A quantitative study of developmental and evolutionary mammalian renal architectonics*. New York: Hoeber Medical Division.

Ortega S, Ittmann M, Tsang SH, Ehrlich M, Basilico C (1998) Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proc Natl Acad Sci U S A* **95,** 5672-5677.

Oshima J, Campisi J (1991) Fundamentals of cell proliferation: control of the cell cycle. *J Dairy Sci* **74,** 2778-2787.

Oxburgh L, Chu GC, Michael SK, Robertson EJ (2004) TGFbeta superfamily signals are required for morphogenesis of the kidney mesenchyme progenitor population. *Development* **131,** 4593-4605.

Ozawa M, Huang RP, Furukawa T, Muramatsu T (1988) A Teratocarcinoma Glycoprotein Carrying A Developmentally Regulated Carbohydrate Marker Is A Member of the Immunoglobulin Gene Superfamily. *J Biol Chem* **263,** 3059-3062.

Pachnis V, Mankoo B, Costantini F (1993) Expression of the c-ret proto-oncogene during mouse embryogenesis. *Development* **119,** 1005-1017.

Paratcha G, Ledda F, Baars L, Coulpier M, Besset V, Anders J, Scott R, Ibanez CF (2001) Released GFRalpha1 potentiates downstream signalling, neuronal survival, and differentiation via a novel mechanism of recruitment of c-Ret to lipid rafts. *Neuron* **29,** 171-184.

Partanen AM (1990) Epidermal growth factor and transforming growth factor-alpha in the development of epithelial-mesenchymal organs of the mouse. *Curr Top Dev Biol* **24,** 31-55.

Perantoni AO, Dove LF, Karavanova I (1995) Basic fibroblast growth factor can mediate the early inductive events in renal development. *Proc Natl Acad Sci U S A* **92,** 4696-4700.

Peter K (1909) *Untersuchungen uber Bau und Entwickelung der Niere.* Jena, Gustav Fischer.

Peters CA (2001) Animal models of fetal renal disease. *Prenatal Diagnosis* **21,** 917-923.

Petrovic S, Wang Z, Ma L, Seidler U, Forte JG, Shull GE, Soleimani M (2002) Colocalization of the apical Cl-/HCO3- exchanger PAT1 and gastric H-K-ATPase in stomach parietal cells. *AJP - Gastrointestinal and Liver Physiol* **283,** G1207-G1216.

Pichel JG, Shen LY, Sheng HZ, Granholm AC, Drago J, Grinberg A, Lee EJ, Huang SP, Saarma M, Hoffer BJ, Sariola H, Westphal H (1996) Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382,** 73-76.

Pihakaski-Maunsbach K, Tokonabe S, Vorum H, Rivard CJ, Capasso JM, Berl T, Maunsbach AB (2005) The {gamma}-subunit of Na-K-ATPase is incorporated into plasma membranes of mouse IMCD3 cells in response to hypertonicity. *AJP - Renal Physiol* **288,** F650-F657.

Piper M, Georgas K, Yamada T, Little M (2000) Expression of the vertebrate Slit gene family and their putative receptors, the Robo genes, in the developing murine kidney. *Mech Dev* **94,** 213-217.

Piper M, Nurcombe V, Wilkinson L, Little M (2002) Exogenous Slit2 does not affect ureteric branching or nephron formation during kidney development. *Int J Dev Biol* **46,** 545-550.

Piscione TD, Yager TD, Gupta IR, Grinfeld B, Pei Y, Attisano L, Wrana JL, Rosenblum ND (1997) BMP-2 and OP-1 exert direct and opposite effects on renal branching morphogenesis. *Am J Physiol* **273,** F961-F975.

Plisov SY, Yoshino K, Dove LF, Higinbotham KG, Rubin JS, Perantoni AO (2001) TGF beta 2, LIF and FGF2 cooperate to induce nephrogenesis. *Development* **128,** 1045-1057.

Pohl M, Sakurai H, Bush KT, Nigam SK (2000) Matrix metalloproteinases and their inhibitors regulate in vitro ureteric bud branching morphogenesis. *AJP - Renal Physiol* **279,** F891-F900.

Porteous S, Torban E, Cho NP, Cunliffe H, Chua L, Mcnoe L, Ward T, Souza C, Gus P, Giugliani R, Sato T, Yun K, Favor J, Sicotte M, Goodyer P, Eccles M (2000) Primary renal hypoplasia in humans and mice with PAX2 mutations: evidence of increased apoptosis in fetal kidneys of Pax2(1Neu) +/- mutant mice. *Hum Mol Genet* **9,** 1-11.

Potter ED (1972) *Normal and abnormal development of the kidney*. Chicago: Year Book Medical publishers Inc.

Qiao J, Cohen D, Herzlinger D (1995) The metanephric blastema differentiates into collecting system and nephron epithelia in vitro. *Development* **121,** 3207-3214.

Qiao J, Sakurai H, Nigam SK (1999a) Branching morphogenesis independent of mesenchymal-epithelial contact in the developing kidney. *Proc Natl Acad Sci U S A* **96,** 7330-7335.

Qiao J, Uzzo R, Obara-Ishihara T, Degenstein L, Fuchs E, Herzlinger D (1999b) FGF-7 modulates ureteric bud growth and nephron number in the developing kidney. *Development* **126,** 547-554.

Qiao J, Bush KT, Steer DL, Stuart RO, Sakurai H, Wachsman W, Nigam SK (2001) Multiple fibroblast growth factors support growth of the ureteric bud but have different effects on branching morphogenesis. *Mech Dev* **109,** 123-135.

Raatikainen-Ahokas A, Hytonen M, Tenhunen A, Sainio K, Sariola H (2000) BMP-4 affects the differentiation of metanephric mesenchyme and reveals an early anterior-posterior axis of the embryonic kidney. *Dev Dyn* **217,** 146-158.

Raj GV, Auge BK, Assimos D, Preminger GM (2004) Metabolic abnormalities associated with renal calculi in patients with horseshoe kidneys. *J Endourol* **18,** 157-161.

Rauchman MI, Nigam SK, Delpire E, Gullans SR (1993) An osmotically tolerant inner medullary collecting duct cell line from an SV40 transgenic mouse. *Am J Physiol* **265,** F416-F424.

Rickard SM, Mummery RS, Mulloy B, Rider CC (2003) The binding of human glial cell line-derived neurotrophic factor to heparin and heparan sulfate: importance of 2-O-sulfate groups and effect on its interaction with its receptor, GFRalpha1. *Glycobiology* **13,** 419-426.

Ritvos O, Tuuri T, Eramaa M, Sainio K, Hilden K, Saxen L, Gilbert SF (1995) Activin disrupts epithelial branching morphogenesis in developing glandular organs of the mouse. *Mech Dev* **50,** 229-245.

Rottier R, Tibboel D (2005) Fetal lung and diaphragm development in congenital diaphragmatic hernia. *Semin Perinatol* **29,** 86-93.

Ruf RG, Xu PX, Silvius D, Otto EA, Beekmann F, Muerb UT, Kumar S, Neuhaus TJ, Kemper MJ, Raymond RM, Jr., Brophy PD, Berkman J, Gattas M, Hyland V, Ruf EM, Schwartz C, Chang EH, Smith RJH, Stratakis CA, Weil D, Petit C, Hildebrandt F (2004) SIX1 mutations cause branchio-oto-renal syndrome by disruption of EYA1-SIX1-DNA complexes. *Proc Natl Acad Sci U S A* **101,** 8090-8095.

Sainio K, Nonclercq D, Saarma M, Palgi J, Saxen L, Sariola H (1994) Neuronal characteristics in embryonic renal stroma. *Int J Dev Biol* **38,** 77-84.

Sainio K, Suvanto P, Davies J, Wartiovaara J, Wartiovaara K, Saarma M, Arumae U, Meng XJ, Lindahl M, Pachnis V, Sariola H (1997) Glial-cell-line-derived neurotrophic factor is required for bud initiation from ureteric epithelium. *Development* **124,** 4077-4087.

Sainio K (2003a) Chapter 19: Experimental Methods for Studying Urogenital Development. *The kidney, from normal development to congenital disease.* Vize PD, Woolf AS, Bard JB. London: Acedemic Press

Sainio K (2003b) Chapter 6: Development of the Mesonephric kidney. *The kidney, from normal development to congenital disease.* Vize PD, Woolf AS, Bard JB. London: Acedemic Press

Sajithlal G, Zou D, Silvius D, Xu P (2005) Eya1 acts as a critical regulator for specifying the metanephric mesenchyme. *Dev Biol* **284,** 323-336.

Sakai T, Larsen M, Yamada KM (2003) Fibronectin requirement in branching morphogenesis. *Nature* **423,** 876-881.

Sakurai H, Barros EJ, Tsukamoto T, Barasch J, Nigam SK (1997) An in vitro tubulogenesis system using cell lines derived from the embryonic kidney shows dependence on multiple soluble growthâfactors. *Proc Natl Acad Sci U S A* **94,** 6279-6284.

Sakurai H, Nigam SK (1998) In vitro branching tubulogenesis: implications for developmental and cystic disorders, nephron number, renal repair, and nephron engineering. *Kidney Int* **54,** 14-26.

Sakurai H, Bush KT, Nigam SK (2001) Identification of pleiotrophin as a mesenchymal factor involved in ureteric bud branching morphogenesis. *Development* **128,** 3283-3293.

Sampogna RV, Nigam SK (2004) Implications of Gene Networks for Understanding Resilience and Vulnerability in the Kidney Branching Program. *Physiol* **19,** 339-347.

Sanchez MP, SilosSantiago I, Frisen J, He B, Lira SA, Barbacid M (1996) Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382,** 70-73.

Sariola H, Holm K, Henke-Fahle S (1988) Early innervation of the metanephric kidney. *Development* **104,** 589-599.

Sariola H, Sainio K, Bard J (2003) Chapter 13: Fates of the metanephric mesenchyme. *The kidney, from normal development to congenital disease.* Vize PD, Woolf AS, Bard JB. London: Acedemic Press

Saxen L (1987) *Organogenesis of the kidney*. Cambridge: Cambridge University Press.

Schmidt-Ott KM, Yang J, Chen X, Wang H, Paragas N, Mori K, Li JY, Lu B, Costantini F, Schiffer M, Bottinger E, Barasch J (2005) Novel regulators of kidney development from the tips of the ureteric bud. *J Am Soc Nephrol* **16,** 1993-2002.

Schmidt C, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, Gherardi E, Birchmeier C (1995) Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* **373,** 699-702.

Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V (1994) Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature* **367,** 380-383.

Schumacher K, Strehl R, Minuth WW (2002) Detection of glycosylated sites in embryonic rabbit kidney by lectin chemistry. *Histochem Cell Biol* **118,** 79-87.

Shah MM, Sampogna RV, Sakurai H, Bush KT, Nigam SK (2004) Branching morphogenesis and kidney disease. *Development* **131,** 1449-1462.

Shakya R, Watanabe T, Costantini F (2005a) The role of GDNF/Ret signalling in ureteric bud cell fate and branching morphogenesis. *Dev Cell* **8,** 65-74.

Shakya R, Jho EH, Kotka P, Wu Z, Kholodilov N, Burke R, D'Agati V, Costantini F (2005b) The role of GDNF in patterning the excretory system. *Dev Biol* **283,** 70-84.

Shannon JM, Nielsen LD, Gebb SA, Randell SH (1998) Mesenchyme specifies epithelial differentiation in reciprocal recombinants of embryonic lung and trachea. *Dev Dyn* **212,** 482-494.

Sharpe J (2003) Optical projection tomography as a new tool for studying embryo anatomy. *J Anat* **202,** 175-181.

Sharpe J (2004) Optical projection tomography. *Annu Rev Biomed Eng* **6,** 209-228.

Srinivas S, Wu Z, Chen CM, D'Agati V, Costantini F (1999a) Dominant effects of RET receptor misexpression and ligand-independent RET signalling on ureteric bud development. *Development* **126,** 1375-1386.

Srinivas S, Goldberg MR, Watanabe T, D'Agati V, al-Awqati Q, Costantini F (1999b) Expression of green fluorescent protein in the ureteric bud of transgenic mice: a new tool for the analysis of ureteric bud morphogenesis. *Dev Genet* **24,** 241-251.

Stark K, Vainio S, Vassileva G, McMahon AP (1994) Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* **372,** 679-683.

Steer DL, Bush KT, Meyer TN, Schwesinger C, Nigam SK (2002) A strategy for in vitro propagation of rat nephrons. *Kidney Int* **62,** 1958-1965.

Steer DL, Shah MM, Bush KT, Stuart RO, Sampogna RV, Meyer TN, Schwesinger C, Bai X, Nigam SK, Esko JD (2004) Regulation of ureteric bud branching morphogenesis by sulfated proteoglycans in the developing kidney. *Dev Biol* **272,** 310-327.

Steiner P, Strehl R, Kloth S, Tauc M, Minuth WW (1997) In vitro development and preservation of specific features of collecting duct epithelial cells from embryonic rabbit kidney are regulated by the electrolyte environment. *Differentiation* **62,** 193-202.

Strehl R, Minuth W (2001) Partial identification of the mab cd Ampl antigen at the epithelial-mesenchymal interface in the developing kidney. *Histochemistry and Cell Biol* **116,** 389-396.

Stryer L (1995) *Biochemistry*. New York: W.H.Freeman and Company.

Stuart RO, Bush KT, Nigam SK (2003) Changes in gene expression patterns in the ureteric bud and metanephric mesenchyme in models of kidney development. *Kidney Int* **64,** 1997-2008.

Suvanto P, Wartiovaara K, Lindahl M, Arumae U, Moshnyakov M, Kuitunen N, Airaksinen MS, Palotie A, Sariola H, Saarma M (1997) Cloning, mRNA distribution and chromosomal localisation of the gene for glial cell line-derived neurotrophic factor receptor beta, a homologue to GDNFR-alpha. *Hum Mol Genet* **6,** 1267-1273.

Tang MJ, Cai Y, Tsai SJ, Wang YK, Dressler GR (2002) Ureteric Bud Outgrowth in Response to RET Activation Is Mediated by Phosphatidylinositol 3-Kinase. *Dev Biol* **243,** 128-136.

Taub M, Wang Y, Szczesny TM, Kleinman HK (1990) Epidermal Growth Factor or Transforming Growth Factor α is Required for Kidney Tubulogenesis in Matrigel Cultures in Serum-Free Medium. *Proc Natl Acad Sci U S A* **87,** 4002-4006.

Torres VE (2004) Therapies to slow polycystic kidney disease. *Nephron Exp Nephrol* **98,** e1-e7.

Treanor JJ, Goodman L, de Sauvage F, Stone DM, Poulsen KT, Beck CD, Gray C, Armanini MP, Pollock RA, Hefti F, Phillips HS, Goddard A, Moore MW, Buj-Bello A, Davies AM, Asai N, Takahashi M, Vandlen R, Henderson CE, Rosenthal A (1996) Characterization of a multicomponent receptor for GDNF. *Nature* **382,** 80-83.

Utriainen A, Sormunen R, Kettunen M, Carvalhaes LS, Sajanti-E, Eklund L, Kauppinen R, Kitten GT, Pihlajaniemi T (2004) Structurally altered basement membranes and hydrocephalus in a type XVIII collagen deficient mouse line. *Hum Mol Genet* **13,** 2089-2099.

Vainio S, Lin Y, Pihlajaniemi T (2003) Induced Repatterning of Type XVIII Collagen Associates with Ectopic Sonic Hedgehog and Lung Surfactant C Gene Expression and Changes in Epithelial Epigenesis in the Ureteric Bud. *J Am Soc Nephrol* **14,** S3-S8.

van Adelsberg J, Sehgal S, Kukes A, Brady C, Barasch J, Yang J, Huan Y (2001) Activation of hepatocyte growth factor (HGF) by endogenous HGF activator is required for metanephric kidney morphogenesis in vitro. *J Biological Chemistry* **276,** 15099-15106.

Vega QC, Worby CA, Lechner MS, Dixon JE, Dressler GR (1996) Glial cell line-derived neurotrophic factor activates the receptor tyrosine kinase RET and promotes kidney morphogenesis. *Proc Natl Acad Sci U S A* **93,** 10657-10661.

Vijayan M, Chandra N (1999) Lectins. *Curr Opin Struct Biol* **9,** 707-714.

Vilar J, Gilbert T, Moreau E, Merlet-Benichou C (1996) Metanephros organogenesis is highly stimulated by vitamin A derivatives in organ culture. *Kidney Int* **49,** 1478-1487.

Vilar J, Lalou C, Duong VH, Charrin S, Hardouin S, Raulais D, Merlet-Benichou C, Lelievre-Pegorier M (2002) Midkine is involved in kidney development and in its regulation by retinoids. *J Am Soc Nephrol* **13,** 668-676.

Vize PD (2003) Chapter 1: Introduction: Embryonic Kidneys and Other Nephrogenic Models. *The kidney, from normal development to congenital disease.* Vize PD, Woolf AS, Bard JB. London: Acedemic Press

Vize PD, Carroll TJ, Wallingford JB (2003) Chapter 3: Induction, Development, and Physiology of the Pronephric tubules. *The kidney, from normal development to congenital disease.* Vize PD, Woolf AS, Bard JB Acedemic Press

Vukicevic S, Kopp JB, Luyten FP, Sampath TK (1996) Induction of nephrogenic mesenchyme by osteogenic protein 1 (bone morphogenetic protein 7). *Proc Natl Acad Sci USA* **93,** 9021-9026.

Waddington CH (1938) The morphogenetic function of vestigial organ in the chick. *J Exp Biol 15,* 371-376.

Walker RA (1989) The use of lectins in histopathology. *Pathol Res Pract* **185,** 826-835.

Wallner EI, Yang Q, Peterson DR, Wada J, Kanwar YS (1998) Relevance of extracellular matrix, its receptors, and cell adhesion molecules in mammalian nephrogenesis. *Am J Physiol* **275,** F467-F477.

Watanabe T, Costantini F (2004) Real-time analysis of ureteric bud branching morphogenesis in vitro. *Dev Biol* **271,** 98-108.

Widenfalk J, Nosrat C, Tomac A, Westphal H, Hoffer B, Olson L (1997) Neurturin and glial cell line-derived neurotrophic factor receptor-beta (GDNFR-beta), novel proteins related to GDNF and GDNFR-alpha with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. *J Neurosci* **17,** 8506-8519.

Wilkie AOM, Slaney SF, Oldridge M, Poole MD, Ashworth GJ, Hockley AD, Hayward RD, David DJ, Pulleyn LJ, Rutland P, Malcolm S, Winter RM, Reardon W (1995) Apert Syndrome Results from Localized Mutations of Fgfr2 and Is Allelic with Crouzon Syndrome. *Nat Genet* **9,** 165-172.

Wilkinson DG (1992) Chapter 6: Wholemount in Situ Hybridisation of Vertebrate Embryos. *In Situ Hybridisation: a Practical Approach.* Wilkinson DG Oxford: IRL Press at Oxford University Press.

Wilkinson DG (2001) Multiple roles of EPH receptors and ephrins in neural development. *Nat Rev Neurosci* **2,** 155-164.

Winnier G, Blessing M, Labosky PA, Hogan BL (1995) Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev* **9,** 2105-2116.

Woolf AS (1997) Multiple causes of human kidney malformations. *Arch Disease in Childhood* **77,** 471-474.

Woolf AS, Kolatsi-Joannou M, Hardman P, Andermarcher E, Moorby C, Fine LG, Jat PS, Noble MD, Gherardi E (1995) Roles of hepatocyte growth factor/scatter factor and the met receptor in the early development of the metanephros. *J Cell Bio.* **128,** 171-184.

Xu PX, Adams J, Peters H, Brown MC, Heaney S, Maas R (1999) Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. *Nat Genet* **23,** 113-117.

Xu PX, Zheng W, Huang L, Maire P, Laclef C, Silvius D (2003) Six1 is required for the early organogenesis of mammalian kidney. *Development* **130,** 3085-3094.

Ye P, Habib SL, Ricono JM, Kim NH, Choudhury GG, Barnes JL, Abboud HE, Arar MY (2004) Fibronectin induces ureteric bud cells branching and cellular cord and tubule formation. *Kidney Int* **66,** 1356-1364.

Yu J, Carroll TJ, McMahon AP (2002) Sonic hedgehog regulates proliferation and differentiation of mesenchymal cells in the mouse metanephric kidney. *Development* **129,** 5301-5312.

Yu J, McMahon AP, Valerius MT (2004) Recent genetic studies of mouse kidney development. *Curr Opin Genet Dev* **14,** 550-557.

Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, Kalluri R (2003) BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nat Med* **9,** 964-968.

Zent R, Bush KT, Pohl ML, Quaranta V, Koshikawa N, Wang Z, Kreidberg JA, Sakurai H, Stuart RO, Nigam SK (2001) Involvement of Laminin Binding Integrins and Laminin-5 in Branching Morphogenesis of the Ureteric Bud during Kidney Development. *Dev Biol* **238,** 289-302.

Zhu L, Skoultchi AI (2001) Coordinating cell proliferation and differentiation. *Curr Opin Genet Dev* **11,** 91-97.

Ziehr J, Sheibani N, Sorenson CM (2004) Alterations in cell-adhesive and migratory properties of proximal tubule and collecting duct cells from bcl-2 -/- mice. *Am J Physiol Renal Physiol* **287,** F1154-F1163.

Acknowledgements

Firstly I would like to thank my supervisor and teacher, Dr. Jamie Davies, for his invaluable guidance and support over the past three years. His great insight and constant help have been very much appreciated.

Thank You to all members (past and present) of the Davies lab who made my Phd learning curve less traumatic. To Jane, Lydia, Mark and Wen for being a constant source of support. I thank you for creating such a friendly atmosphere at work and for providing informative discussion, help and guidance during my time in the lab. To Ms. Linda Wilson from the Confocal facility for her never ending willingness to help, you somehow managed to make the long scanning sessions feel like a pleasure rather than a task. I would like to say thank you also to Anna Shafe for introducing me to the stressful but important world of insitu hybridisation.

To Darren, I admire your ability to put up with my constant moaning and terrible singing and I thank you for never asking to change offices because of it. Thank you for being such a great source of encouragement and enlightenment and always ensuring that I kept it all (be it lab work or other stuff) in perspective.

I have to say a huge Thank You to all my friends in Edinburgh. You have become my second family and I have thoroughly enjoyed my time in Edinburgh because I of you. To Jen, Vicky and Gráinne, I am so lucky that I spent the last three years in the company of such wonderful gals. My only wish is that I could take you all along with me when I go. Thank you always being their when I needed to be cheered up. A special word of thanks goes to Gráinne for giving me a new found appreciation for biostatistics and for never giving up on me on those numerous occasions when I just didn't quite get it. You have the patience of a saint.

Lastly, I would like to thank my family (The Sweeneys, Crowes, Tierneys, (and Kearneys)) at home in Galway for your unwavering love and encouragement over the past number of years. I take it with me wherever I roam.